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THE USE OF P^{32} IN STUDIES ON THE UPTAKE OF PHOSPHORUS BY PLANTS

by SIGURD LARSEN

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In 1923 H e v e s y introduced the use of radioactive isotopes for the investigation of problems in plant and animal physiology [1]). The method has undergone considerable development since that time and artificially produced isotopes are now available in such quantities that experiments involving the use of radioactive fertilizers over a period of months can be carried out.

The present paper describes a fertilizer experiment carried out in the summer of 1949 using three calcium phosphates labelled with P^{32}.

PREPARATION OF THE CALCIUM PHOSPHATES

In order to obtain fertilizers of definite composition and labelled with radiophosphorus the calcium phosphates were synthesied in the laboratory. The methods of preparation were worked out from the recommendations of professor dr. A. T o v b o r g J e n s e n. These were based upon experiences gained in a study of the chemistry of calcium phosphates carried out in the dept. of chemistry under supervision of professor dr. N i e l s B j e r r u m [2]). A detailed description of these methods has not been published previously; since they are more accurate and more convenient than methods hitherto described they are given here.

The chemicals used were 85 per cent phosphoric acid (*pro analysi*) and calcium carbonate (Merck, *pro analysi*). P^{32}, obtained from Oak Ridge, Tennessee, was added as phosphoric acid in an amount corresponding to approximately 0.1 milliCurie per gram phosphorus to the 85 per cent solution of phosphoric acid.

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Primary calcium phosphate.

100 g calcium carbonate were mixed thoroughly in a mortar with 230 g phosphoric acid. During the mixing the following reaction took place:

$$CaCO_3 + 2H_3PO_4 \rightarrow Ca(H_2PO_4)_2, \text{ aq.} + CO_2$$

As the end product still contained calcium carbonate and free phosphoric acid it was recrystallized from a 60 per cent solution of phosphoric acid. The phosphate was added in small portions to the solution which was kept just boiling. When the phosphate was completely dissolved the solution was continuously stirred while being cooled. The primary calcium phosphate crystallized out during the cooling. The mother liquor was separated from the crystals which were washed with acetone and finally airdried.

Secondary calcium phosphate.

The mother liquor and the filtrate from the preparation of primary calcium phosphate were mixed and diluted until a concentration of about 0.2 M with respect to phosphoric acid was reached. Then calcium chloride was added in such an amount as to make the ratio of Ca/P equal to one. This solution along with approximately 0.4 M sodium hydroxide were added slowly while stirring to one litre of a solution of phosphoric acid at a more acid reaction than pH 5.5 By regulating the rate of addition the reaction was kept between pH 5 and 5.5 during the precipitation. The following reaction took place in the vessel:

$$Ca^{++} + H_3PO_4 + 2OH^- \rightarrow CaHPO_4, 2H_2O$$

The crystalline precipitate was washed with icecold water and airdried.

Hydroxyapatite.

3 grammol of secondary phosphate were mixed with 2 grammol calcium carbonate and ground in a ball-mill for 2 hours. The mixture was heated to approximately 1000 centigrade in an electric furnace for 24 hours. During the heating the following reaction took place:

$$3CaHPO_4, H_2O + 2CaCO_3 \rightarrow Ca_5(PO_4)_3OH + CO_2 + 4H_2O$$

Finally the product was ground in a mortar and a powder was obtained.

The composition of the three phosphates was controlled by determining the phosphorus content and the loss by heating. The results are given in table I.

TABLE I

The composition of the calcium phosphates						
	primary		secondary		hydroxyapatite	
	found	calc.	found	calc.	found	calc.
Phosphorus content per cent	24.8	24.6	18.1	18.0	18.4	18.5
Loss by heating per cent	21.7	21.4	26.0	26.1	—	—

ANALYTICAL PROCEDURE

2 g af the dry crop sample were wet digested in a mixture of 4 ml concentrated sulphuric acid and two quantities each of 5 ml of 30 per cent perchloric acid, using an apparatus constructed by Lundblad *et al.* [3]). When the solution was colourless it was diluted, filtered, and neutralized with ammoniawater. Thereafter a mixture of 10 ml of magnesia and ammonium citrate, according to Fiske [4]), and ammonia water were added. The solution was then stirred occasionally, and a precipitate of $MgNH_4PO_4.6H_2O$ formed. After standing overnight the precipitate was collected quantitatively in a small filter which fitted into the counting apparatus. After airdrying at room temperature, the filter with the precipitate was weighed and the phosphorus content of the plant sample calculated. The radiophosphorus content was ascertained by means of a Geiger-Müller tube of the type used by Madsen and Zerahn [5]) connected to an electronic counter and manufactured by Brüel and Kjaer, Nærum, Denmark. The content of radiophosphorus in the phosphorus isolated from the plant was related to the content of radiophosphorus in the phosphorus isolated from the fertilizer. The measurings were carried out at the same time in samples of the same weight. In table II some examples of the analytical data and the calculations are given.

The first 5 columns need no explanations, column no. 6 shows the percentage of phosphorus in the plant originating from the applied fertilizer. The last column will be discussed later in this paper.

TABLE II

Examples (Weight of each sample 2 g dry matter)						
Treatments: m mol PO_4 per kg soil	Precipitate: Magnesium-ammonium-phosphate	P in the samples per cent (grain)	P^{32} in count per min. per 0.1 g P in samples isolated from		C in per cent of C_0	$\left(\frac{C_0}{C} - 1\right) x$
			the plant	the fertilizer		
"x"	mg		"C"	"C_0"		"y"
0	44.1	0.278	0	0	0	—
	43.4	0.274	0	0	0	—
1	52.6	0.332	540	958	55.7	0.78
	54.1	0.342	556	995	55.8	0.79
2	63.4	0.402	828	1152	71.8	0.78
	62.7	0.397	796	1132	70.2	0.84
4	73.7	0.466	1131	1312	86.2	0.64
	73.3	0.463	1098	1305	84.3	0.76

EXPERIMENTAL PROCEDURE

Culture pots each having a surface of 500 sq. cm and a depth of 40 cm, were used in the experiment. The pots are described by Bondorff [6]). Each pot contained 25 kg of a sandy soil from Jutland. The humus content was 2 per cent, pH 6.5, and the phosphate content 0.90 millimol, per kg soil determined according to the method of Damsgaard-Sørensen [7]). All the pots were treated with 10 g potassium nitrate, 0.25 g manganese sulphate, and 0.25 g copper sulphate. The phosphates were added to the soil in the following amounts: the primary and secondary calcium phosphates each at 0, 1, 2, and 4 millimol PO_4 levels and hydroxyapatite at 0, 1, 2, 4, and 8 millimol PO_4 levels per kg soil and thorough mixing was carried out in each case. This manner of application was preferred, as phosphate when applied to the soil surface may not be washed down by rain water but fixed in the upper layer. A further disadvantage of placing the labelled phosphate on the soil surface is that, as the phosphates dissolve and pass in solution through the soil there will be an exchange of phosphate ions between the liquid and the solid phase causing an alteration of the ratio of P^{32} to P^{31} since although both P^{32} and P^{31} will move into the solid phase, only P^{31} will move out. This was demonstrated by means of the following simple experiment: A solution containing known amounts of P^{32} and P^{31} (as

phosphate ions) and an amount of water equivalent to a rain-fall of 200 mm were placed on top of a column of soil saturated with water. After the water had passed through the soil the distribution of the added P^{32} and P^{31} was determined. The results are given in figure 1, from which it is seen that the distribution of the added P^{31} differs from that of the added P^{32}.

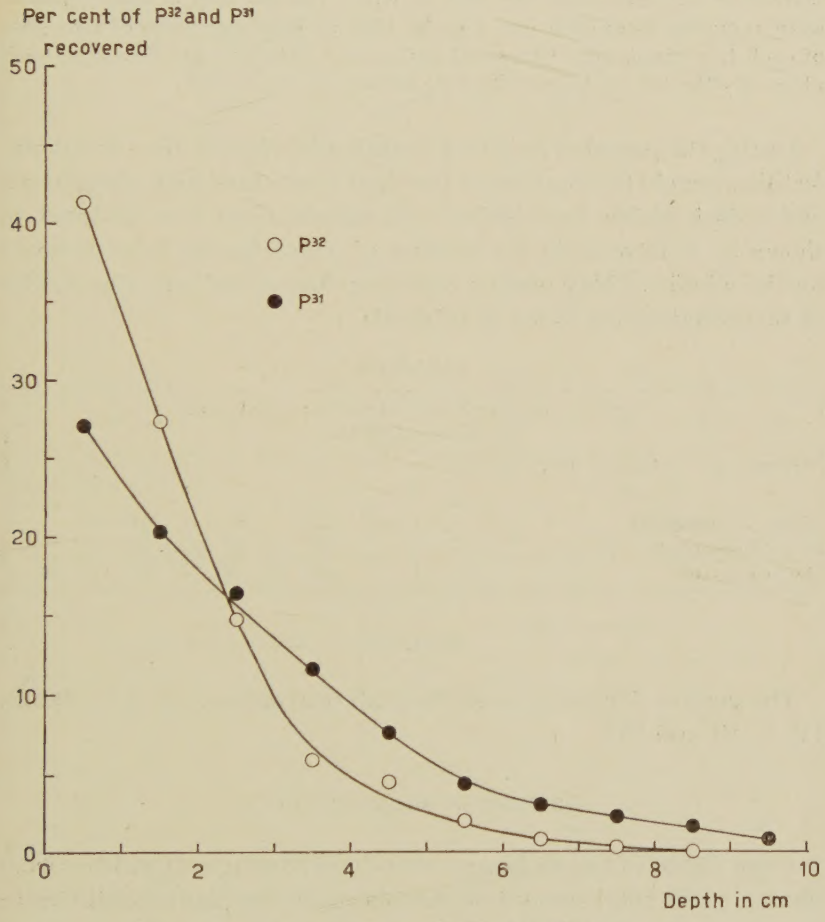

Fig. 1. The distribution of P^{32} and P^{31} in a soil.

By distributing the labelled phosphate throughout the soil a dispersal of the radiation is obtained, and therefore the effect on the plant roots is diminished or possibly avoided. The effect of the radiation on the uptake of phosphorus from fertilizer, as observed by

Russell *et al.*[8]), will also be avoided as the plant cannot take up phosphorus from regions free of radiophosphorus when the labelled phosphates are evenly distributed in the soil.

The soil was fertilized the 20th of April and two days later 35 grains of barley were sown in each pot. The coleoptiles of the seedlings appeared above the soil surface on the 28th of April. On the 10th of May 7 plants were removed from each pot. On the 10th of June the crops of two pots of each treatment were harvested and on the 25th of June flowering took place. By the 6th of August the ripe barley was harvested.

During the period of growth a remarkable effect of the phosphatic fertilizers could be observed as the plant not treated with phosphorus had a dark bluish green colour and development was hindered as shown by a decrease in the number of plants having lateral shoots by the middle of May when a counting was carried out. The results of this counting are given in table III.

TABLE III

Number of plants with lateral shoots per 100 plants by the middle of May					
Treatments, millimols PO_4 per kg soil.	0	1	2	4	8
prim. Ca-phosphate	1	72	91	94	—
sec. Ca-phosphate.	1	85	93	96	—
hydroxyapatite	1	79	92	94	96

RESULTS

The yield of dry matter and the analytical data are given in tables IV, V, VI and VII.

DISCUSSION OF THE RESULTS

From Table VII it can be seen that the percentage of fertilizer phosphorus of the total amount of phosphorus in the plants continues to increase during the 40–50 days after sowing and then remains constant in percentage until the ripe plant is harvested. The most probable explanation of this is that the influence of the phosphorus already present in seed (3.5 mg per pot) is greatest during the first few weeks, then gradually diminishes during the growth, and finally becomes negligible as the uptake of phosphorus increases. In addition an excretion

TABLE IV

The yield of dry matter and the uptake of phosphorus from the soil and the fertilizer by the plant (top), as shown by analysis of the shoots, sampled on the 10th of May and calculated per 25 plants

Exp. no.	Treatments: Ca-phosphate	Treatments: Amount in millimols PO_4 per kg soil	Dry matter per pot	Total P in dry matter	P taken up from the fertilizer per cent of total P	P taken up from: fertilizer	P taken up from: soil + seed
		"x"	g	mg	$\frac{C}{C_0}$ 100 *)	mg	mg
1		0	0.34	1.19	0	0	1.19
2	primary	1	0.59	4.32	23	1.00	3.32
3	primary	2	0.67	5.45	45	2.44	3.01
4	primary	4	0.74	8.25	54	4.46	3.79
5	secondary	1	0.68	4.61	27	1.26	3.35
6	secondary	2	0.67	6.29	54	3.39	2.90
7	secondary	4	0.67	7.33	64	4.67	2.66
8	hydroxy-apatite	1	0.71	3.74	25	0.95	2.79
9	hydroxy-apatite	2	0.79	5.07	43	2.18	2.89
10	hydroxy-apatite	4	0.88	7.00	54	3.77	3.23
11	hydroxy-apatite	8	0.72	6.08	55	3.34	2.64

*) See the text.

TABLE V

The yield of dry matter and the uptake of phosphorus by plants harvested on 10th of June. Treatment (x): see table IV

Exp. no.	Dry matter per pot	Total P in dry matter	P taken up from the fertilizer per cent of total P	P taken up from: fertilizer	P taken up from: soil + seed	$y = \left(\frac{C_0}{C} - 1\right) x$
	g	mg	$\frac{C}{C_0}$ 100	mg	mg	
1	4.0	7.2	0	0	7.2	—
2	23.0	99	56	55	54	0.80
3	24.2	121	73	88	33	0.74
4	25.5	165	84	139	26	0.76
5	23.5	96	55	53	43	0.82
6	28.5	150	70	105	45	0.86
7	28.0	190	80	152	38	1.00
8	20.5	70	43	43	40	1.33
9	25.5	125	62	78	47	1.23
10	28.0	160	70	112	48	1.71
11	27.5	157	70	110	47	3.42

TABLE VI

Yield results and analysis of the ripe barley plants harvested the 6th of August									
Exp. no.	Dry matter in		P in the dry matter			P taken up from fert. per cent of total P	P taken up from		$y = \left(\frac{C_0}{C} - 1\right) x$
	Grain	Straw	Grain	Straw	Total		fert.	soil + seed	
	g/pot	g/pot	mg	mg	mg	$\frac{C}{C_0}$ 100	mg	mg	
1	18.9	27.5	53	20	73	0	0	73	—
2	43.5	62.9	148	58	206	56	115	91	0.79
3	46.0	70.0	184	68	252	71	180	72	0.82
4	51.2	71.0	235	165	400	85	340	60	0.71
5	40.3	64.7	133	60	193	57	105	88	0.82
6	44.9	65.1	170	82	253	67	169	83	0.99
7	50.9	68.0	214	125	339	85	290	49	0.71
8	42.6	55.0	132	30	162	47	76	86	1.13
9	41.0	63.0	148	68	216	62	133	83	1.23
10	56.4	72.5	225	93	318	74	235	83	1.41
11	59.0	70.5	218	130	348	73	258	90	2.93

TABLE VII

The percentage of phosphorus from the fertilizer in the plant phosphorus during the growth period					
Calcium phosphate	Days after sowing	Per cent of fertilizer phosphorus in plant phosphorus			
		Millimol phosphate/kg soil added			
		1	2	4	8
primary	18	23	45	54	—
	49	56	73	84	—
	106	56	71	85	—
secondary	18	27	54	64	
	49	55	70	80	
	106	55	67	85	
hydroxyapatite .	18	25	43	54	55
	49	43	62	70	70
	106	47	62	74	73

of phosphorus, as found by Hevesy *et al.* [9]) and Hevesy [10]), takes place simultaneously with the uptake. This process will also tend to diminish the influence of the seed phosphorus.

When the phosphorus from soil and fertilizers is taken up in a constant ratio it is allowed to assume that the isotope dilution which

takes place if labelled phosphate are added to a soil containing phosphates in exchangeable forms has reached a state of equilibrium. If the soil contains much phosphate in exchangeable forms the phosphorus isolated from the plant clearly will have a low content of radiophosphorus and vice versa. From the amount of phosphate added (x), its content of radiophosphorus (C_0), and the content of radiophosphorus isolated from the plant (C), the amount of the exchangeable phosphorus originally present in the soil can be calculated from the equation:

$$y = \left(\frac{C_0}{C} - 1\right) x$$

This use of isotope dilution is originally applicated in biological investigations as reported by H e v e s y [11]).

If a definite fraction of the soil phosphorus takes part in the isotope dilution the value of the term y will be independent of the amount of phosphate added. From tables V and VI it can be seen that the values of y are nearly constant for both the primary and the secondary calcium phosphates but not for hydroxyapatite which may depend on the solubility of the different phosphates. The two first ones are so easely soluble that an isotope dilution can reach an equilibrium in few weeks; whereas the hydroxyapatite is so slightly soluble that an equilibrium will be established very slowly, may be never. The amount of phosphate which has been found to undergo exchange agrees well with the results obtained by means of D a m s g a a r d-S ø r e n s e n's method for determination of inorganic phosphate in soils: 0.82 (average of prim. and sec.) and 0.90 millimol PO_4 per kg soil, respectively.

It will be seen from tables IV, V and VI that the first treatment increased the utilization of the soil phosphorus by the plants due to its influence upon the development of the plants. In the heavier treatments, however, the utilization of both soil and fertilizer phosphorus decreased according to the law of the diminishing returns, but here also the results from soil treated with hydroxyapatite are different from those obtained in the other treatments. These differences may depend on the physical and the chemical nature of the salt. In contact with the humid soil it forms grains which are not easily dissolved and as a matter of fact grains of that type could still be observed in the soil after harvesting of the plants. Further a

radiation effect cannot be excluded in this case as the radiophosphorus was not evenly distributed through the soil.

SUMMARY

A fertilizer experiment with calcium phosphates labelled with P^{32} was carried out in pots with barley as a test plant. By determining the ratio of P^{32} in fertilizer and plant phosphorus after more than 7 weeks the content of exchangeable phosphate in the soil could be calculated. The results of the calculation were independent of the amount of phosphate added providing it was soluble.

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REFERENCES

1) Hevesy, G., Biochem. J. **17**, 439 (1923).
2) Bjerrum, Niels, Nordisk Naturforskermøde i Helsinki 1936, 329 (1936) and Selected Papers, Copenhagen 1949 (in English).
3) Lundblad, K., Svanberg, O., and Ekman, P., Plant and Soil **1**, 277 (1949).
4) Fiske, C. H., J. Biol. Chem. **46**, 285 (1921).
5) Ambrosen, J., Madsen, B., Ottesen, J. and Zerahn, K., Acta Physiol. Scand. **10**, 195 (1945).
6) Bondorff, K. A., Landbrugets Jorddyrkning I, 13, København 1938.
7) Damsgaard-Sørensen, P., Tids. Planteavl **50**, 653 (1946).
8) Russell, R. S., Adams, S. N. and Martin, R. P., Nature **164**, 993 (1949).
9) Hevesy, G., Linderstrøm-Lang, K., Olsen, C., Nature **137**, 66 (1936).
10) Hevesy, G., Archiv för Bot. **33 A**, 1 (1946).
11) Hevesy, G., Radioactive Indicators, Interscience Publishers, p. 91 (1948).

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METABOLISM OF PLANTS AFFECTED WITH LIME-INDUCED CHLOROSIS (CALCIOSE)

III. MINERAL ELEMENTS

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Previous reports on metabolism of plants affected with lime-induced chlorosis showed the occurrence of profound alteration in nitrogenous constituents (Iljin [19]) and in organic acids and carbohydrates (Iljin [20]). The conditions of unbalance correlated with these changes constitute an index of the course and also the severity of this disease. The present report complements these previous reports by showing the existence of differences between normal and chlorotic foliage in amounts of salts and of certain ash constituents, especially calcium, potassium, magnesium, and iron.

MATERIALS AND METHODS

In much of the work, measurements were made of the salt and mineral element content, using expressed sap. When instead dry material was employed and was extracted by use of water or a weak solution of acetic acid the results did not accord with those obtained by use of sap, mainly for the reason that salts, present in the tissues, may be dissolved by the fluid used as an extractant.

The content of total soluble cations present in the sap was determined both by electrolysis and by chemical analyses. The apparatus used in electrolytic determinations consisted of a vessel in which the cathode, containing mercury, was suspended in a cellophane membrane. The diluted sap was in contact with the anode. By passing a 110-volt electric current through the diluted sap for a sufficient time the cations were deposited and could then be removed leaving the anions in solution. By titration with 0.01 N acid the

anion content was determined, and, on this basis, the total cation content was computed. Usually a somewhat smaller cation content was found by electrolysis than by chemical analysis, but when the data obtained by use of each method were plotted, the two curves tended to parallel each other.

The concentration of cations in saps was calculated as gram-equivalents and afterwards the values were recalculated so as to base the figures on dry weight of leaf tissue.

RESULTS

Total cation content of sap

In the beginning, exploratory analyses were made in attempts to learn whether comparative analyses of saps of normal and chlorotic plants might yield ponderable differences. As a consequence, it was found that the foliage of chlorotic plants possesses a greater content of dissolved salts than does that of normal ones. Data indicating such differences are presented in Table I.

TABLE I

Comparison of salt content of sap of normal and chlorotic foliage

Species	Amount of salts as m equivalents per 1 g of dry weight	
	Normal	Chlorotic
Sambucus nigra	1.1	2.0
Cydonia oblonga	0.7	1.3
Malus domestica	1.0	1.8

Each of the other species tested showed differences in salt content of the magnitude shown in Table I, a result which indicated that it should be of interest to make systematic analyses at intervals throughout the growing season and to correlate concurrently-occurring changes in ash constituents with those in nitrogenous materials and organic acids. It should be mentioned at this juncture that such correlations of the species in Table I showed that the ratio of soluble nitrogen in normal and chlorotic leaves of *Sambucus nigra* was approximately 1 : 2 and that of citric acid 1 : 20. In *Cydonia oblonga* the ratio of soluble nitrogen in normal and in chlorotic leaves was approximately 1 : 3 and that of citric acid 1 : 6; and the ratio of soluble nitrogen in *Malus domestica* was approximately 1 : 4, while that of citric acid was 1 : 14. On the basis of these

preliminary analyses, showing interrelations among constituents, series of samplings were made systematically throughout three growing seasons.

During 1943, healthy and chlorotic apple leaves from each of three varieties, Noble, Bellflower, and Reinette, were gathered at successive intervals of three weeks, to determine comparatively their salt content. The results of such analyses, for the period May 1st to Oct. 5, are shown in Table II.

TABLE II

Total mineral salt content of the sap of normal and chlorotic apple foliage

Variety	Condition	Successive intervals and m equivalents of salts per g of dry weight
Noble	Normal	0.86 — 0.81 — 0.79 — 0.61 — 0.69 — 0.70 — 0.69
	Chlorotic	1.36 — 1.67 — 1.69 — 1.71 — 1.57 — 1.61 — 1.59
Bellflower	Normal	0.87 — 0.99 — 0.95 — 0.97 — 1.04 — 0.91 — 0.88
	Chlorotic	1.00 — 1.16 — 1.06 — 1.31 — 1.39 — 1.40 — 1.13
Reinette	Normal	1.20 — 1.44 — 1.32 — 1.43 — 1.74 — 1.30 — 1.27
	Chlorotic	1.44 — 1.46 — 1.76 — 1.88 — 2.00 — 1.69 — 1.54

The most obvious fact from Table II is that the total salt content of chlorotic leaves exceeds that of normal ones throughout the entire season. Differences of the same magnitude were obtained with these varieties of apples from systematic samplings made in 1944.

Further evidence of the fact that the leaves of trees affected with lime-induced chlorosis contain more salts than normal ones and, in addition, that the more severe the disease the greater the salt content is indicated by results in Table III, from investigations of changes occurring in *Prunus avium*.

TABLE III

Salt content of the sap of leaves of *Prunus avium*

Condition	Successive intervals and ash content, m equivalents per 1 g of dry weight
Normal	0.69 — 0.96 — 0.87 — 0.89
Incipient chlorosis	0.91 — 0.96 — 1.12 — 1.22
Markedly chlorotic	1.42 — 1.20 — 1.23 — —
Very severely affected	1.41 — 1.23 — 1.49 — 1.31

From the findings in the above table the trend toward increase of total salts with increase in severity of chlorosis is clearly defined.

Moreover, it may be added that when chlorosis had progressed to the extent that the leaves were yellowish-white the salt content in occasional samplings was within the range 1.68 to 1.85 m equivalents per g of dry weight.

Systematic analyses were also made of grape foliage during three seasons. The composite results, for the three years, showing separately the maximal, minimal, and average salt content of normal grape leaves are presented in Fig. 1. In comparison selected serial

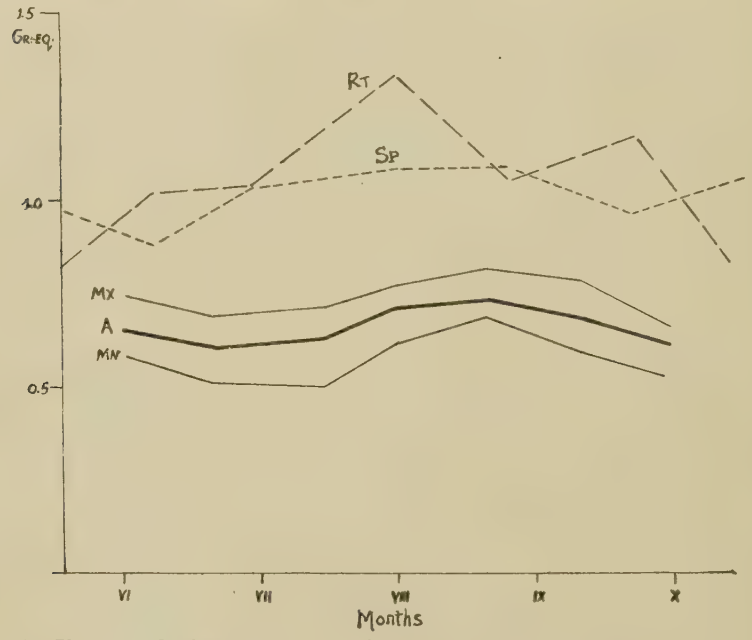

Fig. 1. Changes during the season in concentration of salts in leaves of *Vitic vinifera*. Normal plants: A, average, Mx, maxima; Mn, minima. Chlorotic plants: Rt, the variety Rotgipfler; Sp, the variety Spätrot.

analyses for chlorotic leaves of the varieties Spätrot and Rotgipfler are also shown. The curves showing differences between normal and diseased plants do not parallel each other as might be expected to occur, nor is there parallelism between the curves for the chlorotic species. Whether any significance is to be attached to this lack of relationship is not apparent for it may merely mean lack of control of balance in metabolic processes incidental to the disease.

In general, the findings were similar for all other species analyzed,

notably with *Syringa vulgaris* and *Fraxinus excelsior*. The chlorotic leaves possessed the larger quantities of salts, and the greater amounts occurred always in leaves of plants that were visibly the most severely affected. An additional feature was exhibited, however, by the two species of lupines examined, *Lupinus albus* and *L. luteus*. With *L. albus*, for example, of which analyses of lower leaves and upper leaves were made at successive intervals, using both normal plants and those on soils abundantly supplied with lime, the following results were obtained.

The most obvious difference shown by the results in Table IV is that of a consistently greater salt content of lower leaves of both healthy and diseased plants, that of diseased plants being the greater.

TABLE IV

Comparative salt content of sap of upper and lower leaves of *Lupinus albus*

Condition	Position	Successive intervals and salt content, m equivalents per g of dry weight
Normal	Lower leaves	1.82 — 2.20 — 2.19 — 1.90
,,	Upper leaves	1.31 — 1.75 — 1.64 — 2.20
Chlorotic	Uower leaves	2.11 — 3.99 — 5.15 — 2.43
..	Upper leaves	2.09 — 3.04 — 2.21 — 2.34

The larger content of ash constituents in all chlorotic species studied by the writer accords with findings by Wallace and Mann [37]. Such accumulation of soluble salts is not to be interpreted, however, as a cause of chlorosis but rather as a consequence. For reasons as yet unknown, affected plants, at least in part, lack ability to regulate the intake of salts and also the mechanisms of their utilization in metabolic processes within the tissues. The situation as regards ash constituents is thus entirely in accord with the disturbed balance in metabolism of nitrogenous materials and of organic acids and carbohydrates. It has been suggested (Lindner and Harley [21]) that the proximate cause for disturbance of these processes may be quite alike and may be related to the relatively high level of salts, notably potassium ,which could interfere with iron needed in the enzyme system that is responsible for the formation of chlorophyll.

Potassium in Sap

Several previous investigations, as those by Colin and Grandsire [6]), Wallace and Mann [37]), Wallace [36]),

McGeorge[25]), Iljin[14]), and Lindner and Harley[24]), have shown that the leaves of chlorotic plants have a larger content of potassium than is possessed by those of normal plants. The writer's observations on this point began in 1941, when a few analyses were made of the saps of diseased and healthy leaves of *Robinia pseudacacia*, *Philadelphus coronarius*, *Cydonia oblonga*, *Ailanthus glandulosa*, and *Fraxinus excelsior*. The results of these preliminary random samplings confirmed the finding that chlorosis is accompanied by increase of potassium in the sap, and indicated, in addition, that the potassium content of young leaves, whether in spring or later in the season, is larger than that of older ones, and, in addition, that the largest amounts tend to occur in the most severely chlorotic leaves.

With these observations as a basis, systematic samplings and analyses were undertaken, and, during the growing seasons of 1943 and 1944, series of observations involving apple leaves were made. Typical data from among those secured are presented graphically in Fig. 2.

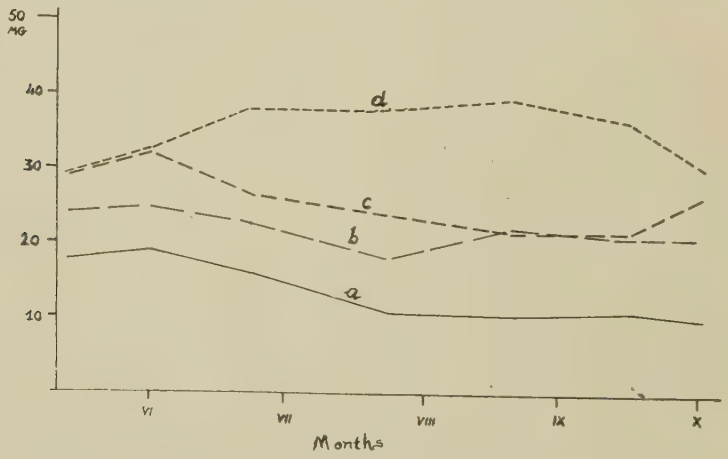

Fig. 2. Changes throughout the season in content of potassium in sap of *Pyrus malus* leaves. a, green leaves; b, weakly chlorotic; c, distinctly chlorotic; d, severely chlorotic.

The most outstanding fact shown by these data is that the leaves of such trees as appear to be most severely affected have the largest content of potassium in the sap. Another feature from the data in Fig. 2 is that the trend of potassium in normal trees is toward

gradual decline throughout the growing season. In diseased trees, however, the course of change is not uniform in rate.

Similar systematic observations were made throughout 1942, 1943, and 1944, of the alterations in potassium content of grape foliage of the three varieties, Rotgipfler, Rheinriesling and Spätrot. The results, graphed in Fig. 3, are summarized in such manner as to portray the important facts.

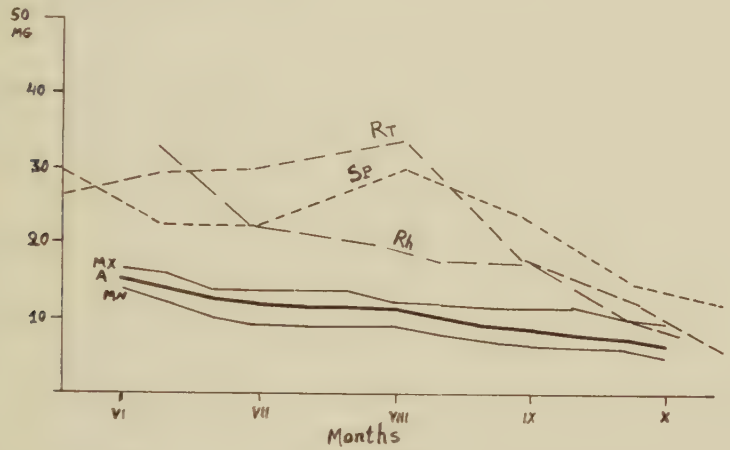

Fig. 3. Alterations in content of potassium in *Vitis vinifera*. Three-year averages, normal leaves; A, general average amounts; M, maximal amounts, Mn, minimal amounts. Chlorotic plants: Rt, the variety Rotgipfler; Rh, Rheinriesling; Sp, Spätrot.

It may be noted, first of all, that three separate lines are shown to represent maximum, minimum, and average quantities, respectively, of potassium in the sap samplings of normal leaves, each line being a three-year average. The other three lines, one for each variety of grape, show that the content of potassium in the sap of diseased leaves exceeds that of normal ones, but that the potassium content of the two groups tends to be approximately alike toward autumn.

Similar analyses made at successive intervals throughout summer involved *Prunus avium*, *Syringa vulgaris*, *Fraxinus excelsior* and *Sambucus nigra*. The trend of changes in potassium, except in the latter species, exhibited no features differing in kind from those with apples and grapes, as given above. There were found only small differences in amounts of potassium in normal and chlorotic leaves of *Sambucus nigra*. This situation may be related to the fact that

magnesium almost completely replaces calcium in this species, as will be pointed out later in this report.

The observations with *Lupinus albus* and *L. luteus*, like those with *Sambucus nigra*, were somewhat aberrant. The analyses definitely showed, however, that the potassium content of chlorotic plants exceeds that of normal ones, and that upper leaves have the greater potassium content.

Calcium and magnesium in sap

In a previous publication, the writer (1942) pointed out that calcium, both as total calcium and as soluble calcium, is present in smaller amount in chlorotic tissue than in normal tissue. In confirmation, Lindner and Harley[24]) found less calcium in the ash of leaves of chlorotic apple and pear trees than in healthy ones. Such results might be interpreted as indicating that plants on limed soils may suffer from a lack of calcium. That such is not necessarily the case for all species of plants is apparent from the present studies.

Among the species analyzed was *Sambucus nigra* to determine its content of calcium in health and in disease. It became apparent early from preliminary analyses, that calcium is normally present in small amount only in this species and that this element is largely replaced by magnesium. The quantity of soluble calcium in sap of normal leaves ranged from 0.1 to 0.4 mg per g of dry weight and that of magnesium from 5.6 to 6.1 mg. In sap from chlorotic plants, however, the calcium content had increased to the range 2.0 to 5.0 mg per g of dry weight and the magnesium to the range 15.5 to 21.6 mg. Throughout 1943 and 1944 systematic samplings at regular intervals confirmed these results with *Sambucus* and showed, in addition, that young leaves have less calcium and magnesium than do old leaves.

Graphs have been prepared from compilation of three season's data bearing on the changes in calcium content and in magnesium content of the sap of grape leaves, and they are shown in Fig. 4.

Obviously, at the beginning of the growing season, the quantity of calcium and magnesium in healthy plants is not too different from that in chlorotic ones, being only slightly less in the former. The subsequent changes, when consecutive analyses are compared, show irregularities, i.e. sometimes an increase and at other times a decrease. As regards healthy plants, however, there is a general

tendency toward gradual progressive increase in amount throughout the season. The fluctuations in content in chlorotic plants indicate that they have lost, in part, their ability to regulate the utilization of calcium and magnesium.

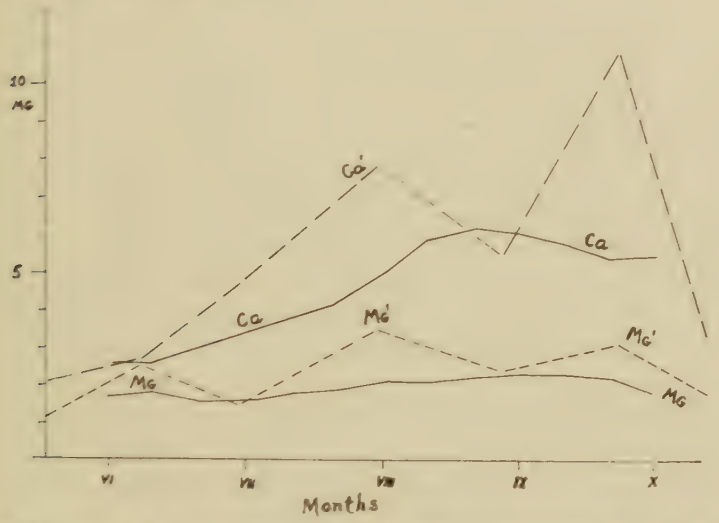

Fig. 4. Changes in soluble calcium and magnesium content of the sap of *Pyrus communis*. Average for three seasons, normal plants. Ca, calcium; Mg, Magnesium. Chlorotic plants, 1943 season. Ca', calcium. Mg', magnesium.

As may be anticipated, a better understanding might be gained of the role of calcium and magnesium in chlorosis if one knew the proportion between the soluble and insoluble portions. To this end, series of analyses of the sap of apples were made, and the data obtained during 1943 are presented in Table V.

It may be explained that the data in Table V do not present as marked contrast between normal and diseased plants as was expected

TABLE V

Percentage of soluble calcium in the sap of apple leaves

Variety	Condition	Intervals and soluble calcium, as per cent of total calcium
Noble	Normal	46 — 25 — 28 — 25 — 25 — 24 — 25
	Chlorotic	67 — 69 — 53 — 62 — 48 — 54 — 49
Beliflower	Normal	48 — 36 — 33 — 35 — 37 — 34 — 39
	Chlorotic	60 — 62 — 39 — 42 — 45 — 49 — 49
Reinette	Normal	61 — 59 — 54 — 44 — 49 — 37 — 40
	Chlorotic	59 — 63 — 64 — 58 — 62 — 55 — 59

because the "so-called" normal Bellflower apple tree proved to be slightly chlorotic and the "so-called" normal Reinette was found to be diseased to a somewhat greater degree. This fact was substantiated from analyses of their nitrogenous constituents and of their content of citric acid, as previously noted, and first became apparent from visible evidence late in 1943. The same trees were observed to be distinctly chlorotic throughout the entire following season. The normal Noble apple tree, however, was healthy and remained so during 1944. It may be seen that its soluble calcium content, which was 46% in the young leaves, soon declined to 25% and then remained practically constant at this level throughout the entire season, independently of the quantity of calcium that may have entered from the soil. The existence of a state of stability or equilibrium between soluble and insoluble calcium is regarded as a phenomenon that characterizes the functioning of healthy plants.

On the other hand, the content of soluble calcium fluctuated throughout the entire season in the diseased apple leaves and never attained a state of equilibrium. Moreover the percentage of soluble calcium in the sap of the leaves of these diseased trees was continuously in excess of that in leaves of normal trees.

A similar relationship of soluble and insoluble calcium was found in sap from normal and chlorotic leaves of *Sambucus nigra*. The insoluble calcium content of normal leaves remained continuously at approximately 90% and that of diseased ones fluctuated between 52 and 69%. Stability of the same pattern was maintained by healthy grape leaves and cherry leaves, the insoluble calcium content of the former being about 82% and that of the latter, about 84%.

Among the other species analyzed which had less insoluble calcium in the leaf sap of diseased plants than in healthy plants are *Ailanthus glandulosa*, *Cydonia oblonga*, *Pyrus communis*, *Cornus mas*, *Fraxinus excelsior*, and *Lupinus albus*.

In previous investigations [14] [15] [18], the writer divided plants into three groups on the basis of the proportion of soluble to insoluble calcium in the sap. One group contains plants having little if any calcium in solution, and includes those in which the accumulating calcium is continuously being precipitated as oxalates. Another group contains plants in which almost all of the calcium remains in solution. The third group, in which is contained the largest number of species, comprises plants in which part of the calcium remains in

solution in varying amounts and the remainder is precipitated as salts. These group differences are inherent within the species, and the reasons for these differences remain unknown. The level at which a balance between soluble and insoluble calcium tends to be maintained in health varies with the species. For example, among the species studied herein, *Sambucus nigra* tends to maintain its precipitated calcium at about 92% of the total calcium content, *Vitis vinifera* at 82%, *Pyrus malus* at 75%, *Syringa vulgaris* at 72%, and *Ailanthus glandulosa* at 50%. When plants are affected with lime-induced chlorosis the equilibria between soluble and insoluble calcium are disturbed, the extent of this disturbance being conditioned by the species, the severity of the disease, and various weather factors.

Ratio of univalent to bivalent elements

The writer, in previous reports [14] [16], pointed out that the ratio of potassium to calcium and of potassium to calcium plus magnesium in plant sap changes throughout the growing season.

It was found that in young tissues there is much potassium and little calcium, and that with age, the relative amount of calcium increases. It, therefore, became of interest to determine whether chlorosis is correlated with disturbance in the continuously changing ratio of potassium to calcium. As a basis to learn whether an intensive study should be initiated, a series of preliminary analyses were made, using *Syringa vulgaris*, cherry, *Robinia pseudacacia*, and *Ailanthus glandulosa*. The results of these analyses were in accord in showing that the ratio of univalent to bivalent ions was different in chlorotic plants from that in normal ones. As a consequence, a study, that extended over three seasons, was begun, using normal grape foliage in comparison with chlorotic foliage, of the varieties Spätrot and Rotgipfler. Pertinent data on the K/Ca ratio are shown graphically in Fig. 5.

It may be noted in Fig. 5 that curve A, an average of values from analyses at 10-day intervals for three seasons, shows a K/Ca ratio of 3.0 on June 1 and of 0.6 on Oct. 1, with a continuous decrease in rate of change of the proportion between potassium and calcium throughout the entire growing season. The K/Ca ratio for the sap of chlorotic Spätrot leaves is the curve Sp., and that of chlorotic Rotgipfler is Rt. The data represented are for the summer of 1943

only. The K/Ca ratio in sap of diseased leaves of each of the two varieties is greater than that of normal leaves. Moreover, there is shown a lack of uniformity in the rates of alteration for both varieties.

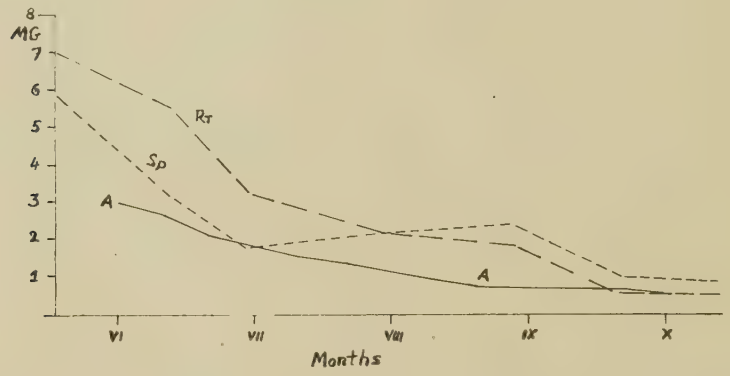

Fig. 5. Changes in K/Ca ratio in the sap of *Vitis vinifera*. A, three-year average, normal plants. Chlorotic plants, 1943 season: Sp, Spätrot; Rh Rheinriesling.

Iron in the sap

Lack of iron in the leaves has long been regarded as a cause of chlorosis. Over one hundred years ago Gris [11]) noted that chlorotic plants became green after having been sprinkled with a solution containing iron salts. Molisch [27]) observed that young leaves, to which iron was applied, may completely recover, whereas recovery was only partial in older leaves. Triwosch [34]), by applying iron salts to the soil around chlorotic lupines, induced recovery, provided the soils did not contain carbonates of calcium and magnesium. Application of ferrosulphate to the soil around cottonwood or to its chlorotic leaves produced little change in older leaves, but newly formed ones became green (Burke [4])).

On the other hand, Heinrich [13]) noted recovery in lupines growing on soils made alkaline with potassium carbonate or made acid with potassium bisulphate or with citric acid. Haas [12]) was able to correlate recovery from chlorosis of citrus trees with changes in pH of the soil. He found calcareous soils to be alkaline at high moisture equivalents but acid at low moisture content.

Ingalls and Shive [21]) correlated pH of tissue fluids with iron content of plants, and Clark [5]), Oserkowsky [29]),

Bennett [2]) and Jacobson [22]) correlated chlorophyll content with iron. The evidence from these and many other investigations supports the conclusion that alkaline soil conditions are essential to production of chlorosis, and that iron is non-available to plants on alkaline soils. In fact, several investigators, among them Fliche and Grandeau [8] [9]), Wallace [36]), and Vidal [35]), found less iron in chlorotic leaves than in green ones. Opposed to this finding are the results of observations by Gile and Carrero [10]), Milad [26]), Wallace and Mann [37]), Allyn [1]), Oserkowsky [30]), and many others. They explain the incidence of chlorosis on whether the iron exists in active or inactive form. In green leaves, iron acts in catalysis of chlorophyll. In chlorotic foliage, they found that little active iron exists.

The apparent contradictions in the foregoing investigations and in many others that bear on chlorosis are the more difficult to explain when one recalls the farmers have so widely and for so many years applied lime with resultant increase in vigor of certain crop plants and increase in abundance of harvest.

The present studies of iron content involve 372 analyses of the sap of leaves, 100 of which were of grape, 56 of apple, 26 of cherry, 73 of white lupine, 61 of yellow lupine, 16 of *Syringa vulgaris*, 16 of *Sambucus nigra*, 15 of *Fraxinus excelsior*, and 9 of *Ailanthus glandulosa*. The average results of these analyses are assembled in Table VI.

The most obvious conclusion that may be drawn from these comparative analyses of the iron content of normal and chlorotic leaves is that this element appears to be present in the leaf sap in ample amount of each of these species. The differences in iron content, between normal leaves and chlorotic leaves does not appear to justify the conclusion that iron *per se* is the cause of chlorosis in these species, since there is complete lack of consistency in balance of iron content, normal foliage having the larger amount of iron in some species, chlorotic foliage in other species, and with little difference in iron content of normal and chlorotic leaves in others. Bennett [2]) concluded that disturbance in iron metabolism in chlorotic plants is intimately related to disturbance in nitrogen metabolism.

TABLE VI

Total-iron content of sap of normal and chlorotic leaves of various species

Species	Condition	Average content as mg/g of dry weight
Ailanthus glandulosa	Normal	0.173
	Chlorotic	0.208
Fraxinus excelsior	Normal	0.094
	Chlorotic	0.096
Sambucus nigra	Normal	0.209
	Chlorotic	0.176
Syringa vulgaris	Normal	0.099
	Chlorotic	0.142
Lupinus albus	Normal	0.238
	Chlorotic	0.187
Lupinus luteus	Normal	0.295
	Chlorotic	0.272
Rotgipfler grape	Normal	0.198
	Chlorotic	0.375
Rheinriesling grape	Normal	0.170
	Chlorotic	0.185
Spätrot grape	Normal	0.274
	Chlorotic	0.362
Knorpel cherry	Normal	0.105
	Chlorotic	0.156
Johannes cherry	Normal	0.119
	Chlorotic	0.149
Noble apple	Normal	0.175
	Chlorotic	0.166
Bellflower apple	Normal	0.119
	Chlorotic	0.128
Reinette	Normal	0.118
	Chlorotic	0.135

Phosphorus in sap

Several investigators, including Pfeiffer and Blanck[31]), Olsen[28]), Clark[5]), Ehrenberg[7]) proposed that phosphates in soils are of great importance in causing iron compounds to become difficultly soluble, and thus in rendering iron non-available. Moreover, at least a part of the phosphorus present within the tissues combines with iron, thus forming precipitates and interfering with distribution or translocation into other organs and tissues. One might expect, therefore, that the heavy application of phosphates, as a component of commercial fertilizer, would favor the production of chlorosis in plants. Brüne and Tacke[3]) and Lieb[23]), however, concluded that plants on limed soils were prevented from becoming chlorotic by the application of phosphates.

In the present study, only limited studies were made of the phosphorus content of leaves, using primarily grape foliage and white lupine foliage.

In the sap of grape leaves, analyses yielded the following quantities of total phosphorus, shown in Table VII.

TABLE VII

Comparative analyses of phosphorus content of normal and chlorotic grape foliage

Condition	Intervals and mg of phosphorus per 1 g of dry weight
Normal	0.39 — 0.29 — 0.28 — 0.25 — 0.27 — 0.27 — 0.21
Chlorotic	0.65 — 0.42 — 0.38 — 0.34 — 0.35 — 0.32 — 0.25

The phosphorus content of the sap of chlorotic grape foliage exceeds that of normal foliage. Such was not the case, however, with white lupine. In consequence, there seems no reason to regard phosphorus as causally related to the development of chlorosis on soils with a high lime content.

DISCUSSION

The foregoing comparative analyses of the mineral element content of normal and chlorotic foliage admittedly is not exhaustive and does not give consideration to content of manganese, boron, copper, zinc, and other essential elements known to be causally related to deficiency diseases of plants characterized by modification in chlorophyll content. Lime-induced chlorosis is not to be confused with these deficiency diseases but is generally considered to be a distinct entity. The differences that have been found herein in constituent salt and cation content between normal and chlorotic foliage clearly demonstrate, in lime-induced chlorosis, that there are correlated modifications in amount of intake and of rate of utilization of certain essential elements. It may be recalled, from previous studies of this series, that the balance between synthesis and analysis of nitrogenous materials in chlorotic plants is disturbed and that the metabolic rate, involving production of organic acids and carbohydrates, is also altered. All of these modifications are taking place simultaneously, all are presumably concatenately interrelated, and it seems reasonable that each should be regarded as a consequence of chlorosis and not as a cause.

Attention may also be directed to the occurrence of an appreciably increased content of water in chlorotic foliage. The writer secured a body of data to support this conclusion, but it is not included

herein, because such data are deemed extraneous to the present report. The cause of such increase in water content, however, must be directly related to accumulation of soluble salts, sugars, or other materials that act to increase the osmotic pressure of the cell sap.

It becomes difficult to postulate a proximate cause of changes in metabolism within leaf tissues so profound as those shown to occur in lime-induced chlorosis. At least, to the writer, such cause is not known, but there appears little reason to regard lime-induced chlorosis as due simply to a deficiency of iron. Moreover, it seems highly probable and, in fact, entirely reasonable to expect that there should be a profound disturbance of all physiologic activities in calciphobic plants when grown as calciphiles. Further support for this conclusion is indicated by Steinberg, Bowling and McMurtrey [33]) from the use of slight excesses of amino acids with tobacco seedlings in aseptic culture. Chlorosis and growth abnormalities resulted from such use. They cautioned against assuming that breakdown of chlorophyll in mineral deficiency diseases may necessarily indicate lack of mineral participation in the formation of chlorophyll, as has for so long been assumed. They postulate that chemical elements may operate in connection with protein metabolizing enzymes.

Apparently the entire problem of mineral deficiency diseases should be given critical re-examination in the light of the findings herein recorded.

SUMMARY

This report deals with changes, associated with lime-induced chlorosis, in the mineral content of leaf sap. Normal and chlorotic leaves saps were compared as to total content of salts and to changes involving content of potassium, calcium, magnesium, iron and phosphorus.

The measurements were made by chemical analyses and by electrolytic methods.

In general, the salt content of the sap of chlorotic leaves exceeds that of normal ones, the more severe the disease the greater the difference. Moreover, the content of salts in normal foliage tends to increase gradually from spring until autumn, whereas there are irregular alterations in salt content of chlorotic leaves throughout the entire growing season. Such lack of uniformity in changes may be attributed to abnormal functioning of mechanisms having to do with intake of minerals and their utilization.

The content of potassium in the sap of chlorotic leaves is the larger. Potassium in normal leaves decreases progressively throughout the growing season, but there is a marked increase in chlorotic ones in midsummer.

Usually chlorotic leaves contain more calcium than healthy ones. In the sap of healthy plants, equilibrium is maintained between soluble and insoluble calcium. In chlorotic plants, however, the proportions fluctuate, the disturbance in balance being greatest in severely diseased plants.

The ratio of potassium to calcium is modified during the course of lime-induced chlorosis and is quite different from that in healthy leaves.

Magnesium occurs in small amounts in each of the species studied, except in *Sambucus nigra*. In this species, the magnesium content increases coincidentally with chlorosis.

The phosphorus content of chlorotic grape leaves is larger than that of normal ones. In other species studied, the changes in phosphorus were not definitive.

The iron content of the sap of some of the species of chlorotic plants exceeded that of normal ones, and was less than that of normal ones in other species. In a few species, the differences were so small as to seem insignificant. Lack of iron *per se* does not appear to be the proximate cause of lime-induced chlorosis.

The entire metabolism of plants affected with lime-induced chlorosis may be assumed to be disturbed, not merely those processes having to do with the formation of chlorophyll and other pigments.

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LITERATURE CITED

1) Allyn, W. F., The relation of lime to the absorption of iron by plants. Proc. Indiana Acad. Sci. **43**, 405–409 (1927).
2) Bennett, J. P., Iron in leaves Soil Sci. **60**, 91–105 (1945).
3) Brüne, F. and Tacke, B., Über das Phosphorbedürfnis der Lupinus auf Heidesandboden. Z. Pflanzenernähr. Düngung Bodenk. **3**, 41 (1924).
4) Burke, E., Chlorosis of trees. Plant Physiol. **7**, 329–334 (1932).
5) Clark, N. A., Green plants and the reactions of iron. Proc. Iowa Acad. Sci. **43**, 185–189 (1936).
6) Colin, H. and Grandsire, A., Mineralisation des feuilles vertes et des chlorotiques. Compt. rend. **181**, 131–135 (1925).
7) Ehrenberg, P., Zusammenfassende Betrachtungen zur Eisenversorgung von Kulturpflanzen. Z. Pflanzenernähr. Düngung **45**, Heft 1–2 (1936).
8) Fliche, P. et Grandeau, L., De l'influence de la composition chimique du sol sur la vegetation du Pin maritime, (*Pinus pinaster Solane*). Ann. chim. phys. Ser. 4. **29**, 383–414 (1873).
9) Fliche, P. et Grandeau, L., De l'influence de la composition du sol sur la vegetation de chataignier. Ann. chim. phys. Ser. 5, **2**, 354–379 (1874).
10) Gile, P. L. and Carrero, J. O., Causes of lime induced chlorosis and availability of iron in the soil. J. Agr. Research **20**, 33–62 (1920).
11) Gris, E., De l'action des compositions ferrugineuses sur la végétation. Ann. chim. phys. Ser. 4, **7**, 201 (1843).
12) Haas, A. R. C., Lime-induced chlorosis of citrus in relation to soil factors. Plant Physiol. **17**, 27–51 (1942).
13) Heinrich, R., Kalk und Lupine. Berlin, 1896.

14) Iljin, W. S., Zusammensetzung der Salze in der Pflanze auf verschiedenen Standorten. I. Kalkpflanzen. Beihefte Botan. Centr. **50**, 95–137 (1932).
15) Iljin, W. S., Boden und Pflanze. Physiologie und Biochemie der Kalk- und Kieselpflanzen. Abhandl. Russ. Forsch. Ges. (Prague) **10**, 76–116 (1940).
16) Iljin, W. S., Die Kalkchlorose der Pflanze und ihre Biochemie. Jahrb. wiss. Botanik, **90**, 464–529 (1942).
17) Iljin, W. S., Biochemische Vorgänge während der Kalkchlorose der weissen Lupine. Ber. deut. botan. Ges. **61**, 138–148 (1943).
18) Iljin, W. S., Salze und organische Säuren bei Kalkpflanzen. Flora **137**, 265–299 (1944).
19) Iljin, W. S., Metabolism of plants affected with lime-induced chlorosis. I. Nitrogen metabolism. Plant and Soil **3**, 239–256 (1951).
20) Iljin, W. S., Metabolism of plants affected with lime-induced chlorosis. II. Organic acids and carbohydrates. Plant and Soil, **3**, 339–351 (1951).
21) Ingalls, R. A. and Shive, J. W., Relation of H-ion concentration of tissue fluids to the distribution of iron in plants. Plant Physiol. **6**, 109–125 (1931).
22) Jacobson, L., Iron in the leaves and chloroplasts of some plants in relation to their chlorophyll content. Plant Physiol. **20**, 233–245 (1945).
23) Lieb, P., Kalkphosphorsäurefactor der gelben Lupine (Lupinus luteus) und seine Beziehungen zum Ertrag dieser Pflanze. Pflanzenbau, A, **3**, 124 (1930).
24) Lindner, R. C. and Harley, C. P., Nutrient interrelations in lime-induced chlorosis. Plant Physiol. **19**, 420–439 (1944).
25) McGeorge, W. T., Potassium in calcareous soils. I. Solubility and availability. II. Some properties of replaceable potassium. Arizona Agr. Exp. Sta. Tech. Bull. **50**, (1933).
26) Milad, Y., The distribution of iron in chlorotic pear trees. Proc. Am. Soc. Hort. Sci. **21**, 93–98 (1924).
27) Molisch, H., Die Pflanze in ihren Beziehungen zum Eisen. Jena, 1892.
28) Olsen, C., Iron absorption and chlorosis in green plants. Compt. rend. trav. lab. Carlsberg, Ser. Chim. **21**, 15–52 (1935).
29) Oserkowsky, J., Hydrogen-ion concentration and iron content of tracheal sap from green and chlorotic pear trees. Plant Physiol. **7**, 253–259 (1932).
30) Oserkowsky, J., Quantitative relation between chlorophyll and iron in green and chlorotic pear leaves. Plant Physiol. **8**, 449–468 (1933).
31) Pfeiffer, Th. and Blanck, E., Die Kalkfeindlichkeit der Lupine, etc. Mitt. landwirt. Inst. Breslau **6**, 273–313 (1911).
32) Rippel, A., The iron chlorosis in green plants caused by manganese. Biochem. Z. **140**, 315–323 (1923).
33) Steinberg, R. A., Bowling, J. D. and McMurtrey Jr., J. E., A possible explanation for symptom formation in tobacco with frenching and mineral deficiencies. Science **110**, 714–715 (1949).
34) Triwosch, S., Über den Einfluss von Magnesium, Eisen und Kalk auf des Wachstum der gelben Lupine. Z. Pflanzenernähr. Düngung Bodenk. **13**, 155–162 (1934).
35) Vidal, J. L., Sur les casues intimes de la chlorose specifique des sols calcaires. Compt. rend. **205**, 1092–1094 (1937).
36) Wallace, T., Investigations on chlorosis of fruit trees. II. The composition of leaves, bark and wood of current season's shoots in cases of lime-induced chlorosis. J. Pomology Hort. Sci. **7**, 172–183 (1928).
37) Wallace, T. and Mann, C. E. T., Investigations on chlorosis of fruit trees. J. Pomology Hort. Sci. **2**, 115–123 (1926).

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VARIATIONS IN THE CONTENT OF PHOSPHORUS, NUCLEIC ACIDS AND ADENINE IN THE LEAVES OF SOME DECIDUOUS TREES DURING THE AUTUMN

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1. INTRODUCTION

The present investigation was undertaken in order to test the applicability of a microbiological method for determining adenine. Leaves of deciduous trees were chosen as test material, since they are known to contain adenine and could be obtained in any desired quantity. When it was found that the content of adenine in the leaves decreased before defoliation, it appeared desirable to make parallel determinations of the amounts of nucleic acids present. This, however, involved a fractionation of the phosphorus-containing compounds of the leaves; the results of these analyses are also fully reported here, as they contribute to the picture of the biochemical changes occurring in the leaves during the autumn.

2. MATERIAL

The leaves to be analysed were taken from the lower branches of trees growing in the Botanical Garden of the University of Uppsala, each species being represented by a single tree. In 1949 series of samples were taken from *Acer platanoides* L., *Tilia platyphylla* SCOP., *Carpinus betulus* L., and *Prunus padus* L., but in 1950, when *Fagus silvatica* L. *f. atropurpurea*, *Quercus petraea* (MATT.) LIEBL., *Sorbus intermedia* (EHRH.) PERS., *Sorbus Aria* L., and *Betula pubescens* EHRH., were tested, only two samples were analysed from each tree. In all cases, at least one sample contained fresh green, in the case of *Fagus* red, leaves, and at least one other sample contained yellow (or otherwise discoloured) leaves which were still attached

to the twigs, though very loosely, so that they could be regarded as being almost equivalent to leaves just detached.

The number of leaves per sample differed according to the species, but in each case represented an average fresh weight of 10 to 20 g. Unusually large or small leaves were avoided, and the leaves selected for each sample were very nearly the average size for the species, though the mean dry weight usually decreased as the leaves became older (Table II).

Immediately after sampling, each leaf was outlined on a weighed sheet of paper in order to estimate the leaf area. The leaves were then dried in an oven at 100°C for 24 hours, weighed, and pulverized in a Turmix blender. The powder thus obtained was used for the following analyses.

3. ANALYTICAL METHODS

a. *The phosphorus fractions*

The fractionation of the phosphorus compounds was performed essentially in accordance with the procedure described by Michael and Heidecker [26] in their study of spinach leaves. This procedure, like most others used for a similar purpose, is based upon the investigation by Kossel [20] and involves extractions of lipid-P by ethanol, and acid-soluble P by hydrochloric acid. The remaining phosphorus is supposed to represent the total nucleic acid P.

Since Euler and Schmidt [11] found that a preliminary treatment with ethanol rendered subsequent extraction with acid more difficult by binding some of the acid-soluble P to the protein it was decided to commence with the acid extraction. In a later experiment, however, it was shown that the order of extraction did not seem to be important for the present material, (Table I*c*, *d*, and *e*). Three successive extractions with ice-cold 0.1 N HCl proved necessary to remove all acid- and water-soluble P from the leaf powder. According to Schmidt and Thannhauser [33], cold trichloroacetic acid might have been more suitable as a solvent, but this appeared to be unsuitable in this case since the extract was to be analysed microbiologically. The extractions were performed as quickly as possible, in order to avoid acid hydrolysis of the nucleic acids (mainly DNA); this procedure did not give higher P values than extraction with trichloroacetic acid (Table I*f*).

Subsequent treatment with absolute ethanol for *c.* 4 hours in a Soxhlet apparatus removed lipid-P.

According to Schmidt and Thannhauser [33], practically all of the phosphorus still remaining in the leaf material could then be extracted by the use of 1 N NaOH at + 37°C for 15 hours, and it was assumed to represent the nucleic acid P. This was supported by the following observa-

TABLE I

The effect of variations in the solvents used and sequence of extractions on the amounts of the phosphorus containing fractions obtained. Similar material (leaves of *Acer platanoides* collected in August 1949) was used in all six experiments. Figures enclosed in brackets indicate the sequence of the extractions, those without brackets the quantity of phosphorus in mg per g dry matter

Solvent	Fraction	Expt. *a*	Expt. *b*	Expt. *c*	Expt. *d*	Expt. *e*	Expt. *f*
Hcl I		(2) 4.95	(1) 4.86	(2) 4.58	(1) 5.26	(1) 5.08	—
HCl II		(3) 1.00	(2) 0.85	(3) 1.05	(2) 0.68	(2) 1.00	—
HCl III	Acid soluble	(4) 0.16	(3) 0.14	(4) 0.19	(3) 0.13	(3) 0.19	—
TCA (5°C) I	P	—	—	—	—	—	(2)
TCA (5°C) II		—	—	—	—	—	(3) } 5.92
TCA (5°C) III		—	—	—	—	—	(4)
Ethanol	Lipid-P	(1) 0.46	(4) 0.56	(1) 0.56	(4) 0.65	(4) 0.54	(1) 0.50
NaOH	Nucleic acid	—	—	(5) 0.79	(5) 0.70	(5) 0.70	(5) 0.69
TCA (90°C)	P	(5) 0.71	(5) 0.68	—	—	—	—
—	Residual P	(6) 0.03	(6) 0.05	(6) 0.05	(6) 0.02	(6) 0.03	(6) 0.03
	Total P	7.31	7.14	7.25	7.47	7.54	7.14

tions. Two parallel extractions were made on the same leaf material using warm (+ 90°C) trichloroacetic acid (Schneider [31]) instead of NaOH, and the results agreed well (Table I*a*, *b*, and *c*). The content of pentose in the trichloroacetic acid extract was estimated by Mejbaum's [21] method and found to be roughly what might be expected from the amount of P present, if the nucleic acids were assumed to have approximately the same composition as ordinary yeast nucleic acid. Efforts were also made to extract the nucleic acids directly from the ethanol-treated material. Williams' [44] method of extraction with NaCl, followed by precipitation of the nucleic acid by acidifying the extract with HCl at a low temperature, was not successful; the precipitation being incomplete. The possibility of using La-salts to bring about precipitation (Caspersson, Hammarsten and Hammarsten [3]), Davidson and Waymouth [4]) was not tested, since the La-precipitation of nucleic acids has proved to be unsatisfactory under certain circumstances (Davidson and Waymouth [5]). The rather complicated method of Hammarsten [15] involving extraction with very concentrated solutions of NaCl + $(NH_4)_2SO_4$ and urea, followed by a precipitation by Cu-salts, was tried in a single case (*Acer platanoides* VIII). The value obtained for the P content of the precipitate agreed almost exactly with that obtained from the NaOH extract.

The residue from the alkali-extraction contained only traces of P and these could be ignored in the accounts of the analyses.

Therefore the fractionation procedure, finally adopted was as follows:

1 g of the leaf powder was suspended in 25 ml 0.1 N HCl (+ three drops of triacetin) in a centrifuge tube at +5°C. After 2 hours the suspension was centrifuged, the supernatant liquid decanted, and the residue again extrac-

ted with two further quantities of HCl. After the third extraction the residue was washed with distilled water, the washings being added to the combined extracts in order to bring them up to a volume of 100 ml. This represented the acid-soluble P fraction.

The residue was then dried at +80°C and transferred to an extraction thimble and extracted in a Soxhlet apparatus with c. 100 ml absolute ethanol for 5 hours. This extract, containing the lipid-P, was concentrated to 25 ml by evaporation.

The residue now remaining was again dried at +80°C and then transferred to a centrifuge tube, along with 25 ml 1 N NaOH, where it remained over night at +37°C. After centrifuging the supernatant, was decanted, the residue washed repeatedly with water, and the washings added to the extract; this fraction contains the nucleic acid P.

The P determinations were carried out according to the method of Teorell[41]).

b. *The determination of adenine*

In the first instance the amount of adenine present in the HCl extract was determined. However, when it was leamed that Schmidt and Thannhauser[33]) had demonstrated that there is a considerable liberation of purines from nucleic acids by treatment with dilute hydrochloric acid, it was considered safer to use another solvent for the extraction of adenine from the leaf material. Contrary to statements in the literature (Levene and Bass[22]), Winterstein and Somló[43]) and others), adenine proved to be fairly soluble in absolute ethanol, this solvent was therefore employed for the extraction. It was found that 100 ml ethanol was more than sufficient for 0.5 g leaf powder since 10 mg adenine dissolve easily in this volume and the quantity of adenine present in the sample tested is always much smaller than that. The extraction was performed in a Soxhlet apparatus.

Quantitative determinations were made using the combined chromatographic and microbiological technique previously described by Fries and Björkman[13]) and Fries and Forsman[14]). The ethanol extract was concentrated to 25 ml, and 1 ml of the concentrate "spotted" on a filter paper strip. *Ophiostoma* mutants were used as test strains, no. 1174 (requiring adenine) for the localisation of the adenine spot, and no. 870 (requiring adenine or hypoxanthine) for the quantitative estimations.

4. RESULTS

The results of the analyses are given in the first instance in terms of P per gram dry weight of leaf powder. Comparisons between values obtained at various times of the season cannot, however, be based on this unit, since it is well-known that the dry weight of leaves often changes significantly with time. It is therefore preferable to express

the P content in terms of mg (or g) per unit area as used by Ramann[30]), Echevin[9])[10]), and Samson and Samisch[32]) or mg per leaf, as used by Deleano and Andreesco[6]), Deleano and Bordeianu[7]), and Olsen[29]). In this work the first method has been used in most cases, i.e. the amount of P in mg per m^2 of leaf. It should perhaps be pointed out that the leaf area refers only to one, and not both leaf surfaces. The second method has been applied only in those cases (*Prunus padus* and *Betula pubescens*) where the small size of the leaves rendered a careful determination of the surface too time-consuming and uncertain.

The constancy of the leaf area and the decrease in dry weight per leaf or per unit area of leaf during the period before defoliation, is shown from Table II. In only two species viz., *Prunus padus* and *Quercus petraea*, were such decreases not observed.

TABLE II

Average values for area, dry weight and phosphorus content of leaves together with the average dry weight of 1 m^2 leaf area

Species	Leaf color [1])	Date of collecting [2])	Average area of leaf in cm^2	Average dry weight of leaf in mg	Dry weight of 1 m^2 leaf area in g	Phosphorus per leaf in mg mg
Acer platanoides	g	18.VIII–6.X	—[3])	—[3])	65.3	—[3])
	y	15.X–22.X	—[3])	—[3])	50.1	—[3])
Tilia platyphylla	g	18.VIII–24.IX	76	474	62.2	1.78
	y	15.X–22.X	65	369	57.2	1.05
Carpinus betulus	g	18.VIII–6.X	23	123	52.7	0.29
	y	22.X	24	104	43.3	0.20
Prunus padus	g	18.VIII–6.X	—	120	—	0.52
	y	6.X–15.X	—	121	—	0.53
Fagus silvatica f. atrop.	r	31.VIII	36	320	88.1	0.52
	b	10.X	34	221	64.2	0.38
Quercus petraea	g	31.VIII	49	418	85.5	1.59
	b	10.X	50	420	83.6	1.29
Sorbus intermedia	g	31.VIII	37	402	107.8	1.74
	yb	18.X	32	258	82.2	1.05
Sorbus Aria	g	31.VIII	31	277	88.6	1.58
	yb	18.X	37	261	69.5	1.31
Betula pubescens	g	31.VIII	—	99	—	0.23
	v	1.X	—	60	—	0.18

[1]) b = brown; g = green; r = red; y = yellow.

[2]) In the case of the first four species the dates refer to 1949, in the case of the last five to 1950.

[3]) Number of leaves too small to permit a calculation of average values.

The amount of P per m^2 of the green leaves was found to vary with species (Table III). The highest values were found in *Acer platanoides* and the two *Sorbus* species, the lowest in *Carpinus betulus* and *Fagus silvatica*. There did not seem to be any correlation between the content of P and the thickness, or rather heaviness, of the leaves. Since only one representative of each species was studied it is impossible to decide whether the differences observed are characteristic of a species or merely differences between individuals.

As seen from Tables III and IV, the acid-soluble P accounts for the major part of the differences between the trees. The values for the amount of lipid-bound P show a remarkable degree of constancy c. 20 to 30 mg per m^2; similarly the nucleic acid P varies comparatively little from one specimen to another, viz., 25 to 50 mg per m^2, in the case of the green leaves. *Carpinus betulus* represents an exception in possessing much smaller quantities of both forms of organically-bound phosphorus, a fact which may be correlated with the thinness and low total phosphorus content of the leaves of this tree.

Most of the phosphorus (c. 60 to 90%) in the leaves obviously occurs as inorganic phosphate and organic phopshoric esters. The ratio, lipid-P/nucleic-acid-P, seems to be fairly constant in the green laeves, the highest value (1.15) being found in *Tilia*, the lowest (0.52) in *Quercus*.

As soon as the first signs of discoloration appear — sometimes even earlier — certain changes occur in the distribution of the phosphorus in the leaves. The percentages of lipid phosphorus and nucleic acid phosphorus decrease; this is followed by a corresponding relative increase in the percentage of acid soluble P (Fig. 1). On the other hand, however, the total amount of phosphorus per m^2 (or per leaf) diminishes in many cases, which means that the actual amount of P per unit area of leaf decreases in each of the phosphorus containing fractions. In *Acer platanoides*, *Carpinus betulus*, and *Prunus padus* no such decrease was observed, while in *Fagus silvatica*, *Quercus petraea*, and *Betula pubescens* the change was perhaps too small to be considered significant. In *Tilia platyphylla* the total phosphorus content dropped to 60% of the original value, relatively large decreases were also found in the two *Sorbus* species. As might be expected, these biochemical changes started at different times in the different cases, obviously at the same time as, or perhaps somewhat earlier than the first signs of discoloration were observed.

TABLE III

Phosphorus content of the various phosphorus containing fractions [1]) in mg per g dry matter and mg per m² of leaf area

ecies	Sample no.	Date	Leaf colour[2])	Number of leaves	Mg P per g dry weight				Mg P per m² leaf area			
					ASP	Lip-P	NAP	Total P	ASP	Lip-P	NAP	Total P
	I	18.VIII	g	9	5.15	0.49	0.67	6.31	306	29	40	375
	II	27.VIII	g	6	6.71	0.44	0.73	7.88	385	25	42	452
	III	7.IX	g	7	6.87	0.67	0.68	8.22	363	35	36	434
plat.	IV	14.IX	g	10	7.17	0.47	0.73	8.37	437	28	44	509
	V	24.IX	g	9	7.27	0.55	0.78	8.60	472	36	51	559
	VI	6.X	g	13	8.58	0.57	0.75	9.90	456	30	40	526
	VII	15.X	gy	13	10.93	0.33	0.45	11.71	493	15	20	528
	VIII	22.X	y	12	7.29	0.22	0.24	7.75	396	12	13	421
	I	18.VIII	g	11	2.76	0.47	0.40	3.63	177	30	26	233
	II	6.IX	g	14	3.01	0.48	0.43	3.92	183	29	26	238
a plat.	III	24.IX	g	12	2.92	0.33	0.42	3.67	183	20	26	229
	IV	15.X	gy	15	2.61	0.33	0.26	3.20	158	20	16	194
	V	22.X	y	25	2.23	0.19	0.19	2.61	124	10	10	144
	I	18.VIII-	g	29	1.25	0.30	0.36	1.91	62	15	18	95
	II	6.IX	g	20	1.72	0.27	0.35	2.34	86	14	18	118
pinus	III	24.IX	g	22	1.85	0.29	0.42	2.56	103	16	23	142
tul.	IV	6.X	g	42	2.09	0.24	0.36	2.69	115	13	20	148
	V	15.X	gy	54	2.12	0.26	0.32	2.70	104	13	16	133
	VI	22.X	y	34	2.50	0.15	0.15	2.80	107	7	7	121
us silv.	I	31.VIII	r	23	0.91	0.31	0.40	1.62	81	27	35	143
	II	10.X	b	23	1.48	0.14	0.12	1.74	85	9	8	112
rcus	I	31.VIII	g	17	2.77	0.27	0.51	3.55	258	23	44	325
aea	II	10.X	b	16	2.61	0.25	0.21	3.07	218	21	18	257
bus	I	31.VIII	g	22	3.74	0.22	0.37	4.33	403	24	40	467
rm.	II	18.X	yb	31	3.71	0.16	0.16	4.03	305	13	13	331
bus	I	31.VIII	g	21	4.79	0.33	0.59	5.71	425	29	52	506
a	II	18.X	yb	21	4.58	0.23	0.24	5.05	318	16	17	351

ASP = acid soluble phosphorus; Lip-P = lipid-phosphorus; NAP = nucleic acid phosphorus.
See Table II.

TABLE IV

Phosphorus content of the various phosphorus containing fractions [1]) in mg per g dry matter and γ per leaf

ecies	Sample no.	Date	Leaf colour[2])	Number of leaves	Mg P per g dry weight				γ P per leaf			
					ASP	Lip-P	NAP	Total P	ASP	Lip-P	NAP	Total P
	I	18.VIII	g	42	3.87	0.38	0.43	4.68	496	49	55	600
	II	27.VIII	g	42	2.88	0.42	0.43	3.73	347	51	52	450
nus	III	6.IX	g	43	3.94	0.24	0.44	4.62	434	27	49	510
adus	IV	29.IX	g	41	5.97	0.30	0.29	6.56	491	25	24	540
	V	6.X	y	43	4.40	0.13	0.24	4.77	480	14	26	520
	VI	15.X	y	72	3.79	0.13	0.27	4.19	489	17	34	540
ula pub.	I	31.VIII	g	59	1.91	0.28	0.36	2.55	173	25	32	230
	II	1.X	y	80	2.67	0.20	0.15	3.02	159	12	9	180

and [2]) Abbreviations explained in Table II and III.

It may also be remarked that each of the three phosphorus containing fractions was present in the leaves right up to the time of leaf fall, although the lipid and nucleic acid P had usually dropped

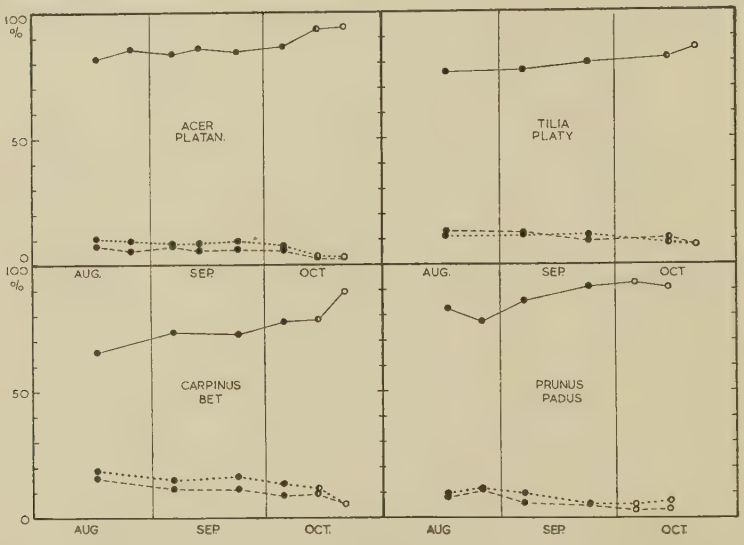

Fig. 1. The distribution of phosphorus among the three phosphorus containing fractions, of the leaf acid-soluble P (continuous lines), lipid-P (broken lines), and nucleic acid P (dotted lines), as percentages fo total phosphorus. The shaded circles refer to green, the circles to yellow, leaves.

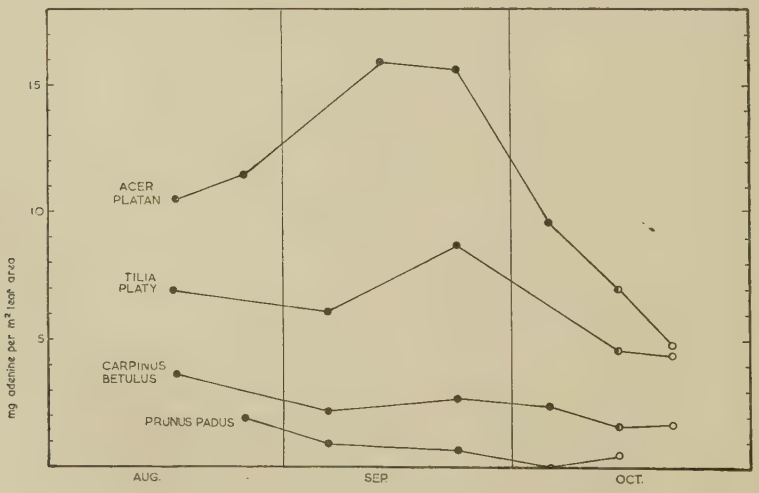

Fig. 2. Amounts of extractable adenine in the leaves from four trees. The shaded circles refer to green, the circles to yellow, leaves.

to c. 30% of the amounts present in the fresh green leaves. The lipid-P of the oak leaves seemed to represent a curous exception, since it did not undergo any change before defoliation.

It will be observed from Fig. 2, that the quantities of adenine which could be extracted with absolute ethanol varied in the different trees. The leaves of *Acer platanoides* were obviously richest in adenine, those of *Prunus padus* poorest, among the species tested. Two further cases not reported in Fig. 2 are *Fagussilvatica* and *Quercus petraea*, material of which was gathered August 31 and October 18, 1950. In *Fagus* were found 8.8 mg and 2.6 mg adenine, respectively; in *Quercus* 4.4 mg and 2.0 mg, respectively, per m^2 leaf area.

In all cases a gradual decrease in the content of extractable adenine was observed (in the case of *Acer platanoides* this occurred after a pronounced maximum in September). This decrease obviously occurred simultaneously with other biochemical changes in the leaves which are characteristic of the discoloration period.

5. DISCUSSION

The question of a partial return to the stem of certain mineral constituents of the leaves prior to defoliation has been dealt with in a great number of investigations from the middle of the last century (Staffel 1850 [39]) up to the present. The existence of such a "back-migration" appeared to be a well established fact after 1914, when Swart [40]) critically discussed the older literature and performed convincing analyses on various plants. Later investigations, particularly those of Echevin [9]) [10]), Deleano and Andreesco [6]), Deleano and Bordeianu [7]), and Olsen [29]), have confirmed these results, and it now seems to be beyond doubt that in most trees and shrubs nitrogen at least passes back to the branches and the stem to a very great extent before defoliation. Other elements behave in different ways in this respect, although a decrease in the content of phosphorus and potassium is obviously a very common phenomenon preceding the withering of leaves in perennial plants.

As regards the amount of phosphorus in the leaves, the results of the present study indicate a certain decrease of this element before the leaf fall. This is very pronounced in *Tilia platyphylla*, but insignificant and uncertain in some other species. In three cases, viz. *Acer platanoides*, *Carpinus betulus* and *Prunus padus*, the amount of phosphorus per unit area remained practically constant.

An interesting question which, as far as is known, does not seem to have been considered, is to what extent the differences found between single trees regarding the extent of back-migration of certain elements from the leaves to the stem are characteristic of the species or merely of the individuals. The quite contradictory results, obtained in the case of phosphorus translocation in beech, obtained by such careful workers as É c h e v i n [9]) and O l s e n [29]), points to the latter alternative. It also seems reasonable to assume that such a process of translocation from leaf to stem may be influenced by climatic and other external factors.

Simultaneously with the discoloration, preceding the fall of the leaves, a transformation of the phosphorus compounds takes place which renders most of the insoluble organically-bound phosphorus soluble in water or weak acids. This process was observed in all trees studied. This resulted in the nucleic acid P decreasing to 20–40%, and the lipid-P to 30–50%, of the original values (except in *Quercus petraea*). É c h e v i n [9]) who distinguished between "soluble P" and "insoluble P", and O l s e n [29]) who separated "sap P" and "non sap P" obviously demonstrated the same phenomenon in beech. This process is connected with the general breaking down of many organic constituents of the leaves, e.g., carbohydrates (O l s e n [29])), proteins (S c h u l z e and S c h ü t z [37])) and nucleic acids (T o n z i g [42])).

It is interesting to note, however, that the falling leaf, despite these processes of translocation and destruction, contains a not inconsiderable quantity of nitrogenous organic constituents valuable for the organisms on and in the soil below the tree. Even with due regard to the uncertainty of the determinations of nucleic acid P, one can conclude that the falling leaves of all the species analysed contain from 50 to 200 mg of nucleic acid per m^2 leaf-area (the nucleic acids being assumed to contain c. 9% P).

One of the nucleic acid constituents, viz., adenine, also occurs in the leaves in the free state or in a very loosely-bound form. Quantitative determinations showed that the content of adenine varied in the different trees, *Acer* giving 10 to 20 mg, *Tilia* c. 7 mg, *Carpinus* c. 3 mg, *Fagus* c. 9 mg, and *Quercus* c. 4 mg per m^2 leaf area. In *Prunus padus* only traces of adenine were found. These values dropped to about half when the leaves turned yellow. From this it may be inferred that the decomposition of nucleic acids did not

simultaneously give rise to an accumulation of liberated adenine. The disappearance of adenine is probably due to the activity of oxidizing enzymes which in the ageing leaves transform adenine into hypoxanthine and guanine into xanthine (Tonzig[42]).

A full-grown beech produces about 5.56 kg leaves, dry weight, per year according to Burger[2]). Assuming they have the same composition as that found in the present study, the falling leaves of this beech tree contain a total of 7.4 g nucleic acids and 0.22 g adenine. The beech forest studied by Burger contained 572 trees per ha, which means that every m^2 of the soil surface received 423 mg of nucleic acids and 13 mg of adenine. Similar calculations based upon the data of Ebermayer[8]), Lindquist[23]), Knudsen and Mauritz-Hansson[18]), Romell[31]), and others, show that the amounts of nucleic acids deposited on the soil of forests every year in the leaves from the trees can usually be reckoned in hundreds of milligrams per m^2.

These facts may have a certain ecological significance for several reasons. It has long been known that nucleic acids and their constituents normally occur in soil, especially in humus, where they seem to be in the form of insoluble and very stable complexes with lignin and proteins (Kaila[17]). It is 40 years since adenine, hypoxanthine, and cytosine were detected in soil hydrolysates (Schreiner and Shorey[35]), Shorey[38]). Later Mockeridge[27]) isolated fairly large amounts of adenine, guanine, uracil and cytosine, together with small quantities of nucleic acid and dinucleotides from various soils and composts *i.a.*, from leaf-mould. The falling leaves obviously form an important source of these soil nucleic acids.

These complex organic materials in litter and soil represent a considerable quantity of nitrogen and phosphorus which is unavailable for higher lpants and becomes available only through the activity of certain microorganisms (Koch and Oelsner[19]), Kaila[17]), and others). On the other hand their simpler constituents may play a role as growth factors for various organisms. The nature of the influence of the purine bases on higher plants is not clear, but some authors have reported a favourable effect of guanine and hypoxanthine (Schreiner and Skinner[36])), as well as of adenine (Bonner and Bonner[1])), particularly on the growth of roots. However, other investigators have not been able to confirm these results (Jones[16]), Kruyt and Veldstra[21])),

The effects of these substances on microorganisms are more conspicuous, for example several bacteria require purines or pyrimidines as essential of stimulating growth-factors. In contrast to the mycorrhiza-forming *Hymenomycetes* (Melin[25]), Norkrans[28])), some litter- and woodinhabiting fungi of the genera *Polyporus* and *Mycena* respond very strongly to the presence of nucleic acid purines, even in very low concentrations (Fries[12])). The varying content of adenine and other nucleic acid components may therefore be a factor controlling the occurrence of various microorganisms in litter and soil.

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SUMMARY

The content of acid soluble, lipid-bound and nucleic-acid phosphorus was determined in leaves collected from 9 species of deciduous trees (*Acer platanoides, Tilia platyphylla, Carpinus betulus, Prunus padus, Fagus silvatica f. atropurpurea, Quercus petraea, Sorbus intermedia, Sorbus Aria*, and *Betula pubescens*) during the autumn. The movement of phosphorus back to the branches and the stem from the leaves was demonstrated in some cases, (this was particularly noticeable in the case of *T.p.*, *S.i.* and *S.A.*). At the same time a transformation of organically bound to inorganic phosphorus occurred in all species. Even in the falling leaves, however, there always remained a certain percentage of nucleic acid P and lipid-P.

In 6 species (*A.p.*, *T.p.*, *C.b.*, *P.p.*, *F.s.*, and *A.p.*) the changes in the content of adenine were also studied, the adenine being determined microbiologically. Before leaf fall a decrease to c. 50% of the original amount was noted.

An approximate calculation has been made of the a mounts of nucleic acids and adenine received by the soil in decidance forests from the fallen leaves each autumn.

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REFERENCES

1) Bonner, D. M. and Bonner, J., On the influence of various growth factors on the growth of green plants. Am. J. Botany **27**, 38–42 (1940).

2) Burger, H., Holz, Blattmenge und Zuwachs. IV. Ein 80jähriger Buchenbestand. Mitt. schweiz. Anst. f. d. forstl. Versuchswesen **21**, 307–348 (1940).

3) Caspersson, T., Hammarsten, E. and Hammarsten, H., Interactions of proteins and nucleic acid. Trans. Faraday Soc. **31**, 367–389 (1935).
4) Davidson, J. N. and Waymouth, C., Tissue nucleic acids. I. Ribonucleic acids and nucleotides in embryonic and adult tissues. Biochem. J. **38**, 39–50 (1944).
5) Davidson, J. N. and Waymouth, C., Tissue nucleic acids. III. The nucleic acid and nucleotide content of liver tissue. Biochem. J. **38**, 379–385 (1944).
6) Deleano, N. T. and Andreescо, M. I., Beiträge zum Studium der Rolle und Wirkungsweise der Mineral- und organischen Stoffe im Pflanzenleben. I. Der quantitative Stoffwechsel der Mineral- und organischen Substanzen in den *Salix fragilis*-Blättern während ihrer Entwicklung. Beitr. z. Biol. d. Pflanzen **19**, 249–286 (1932).
7) Deleano, N. T. and Bordeianu, C., Beiträge zum Studium der Rolle und Wirkungsweise der Mineral- und organischen Stoffe im Pflanzenleben. II. Der quantitative Stoffwechsel der Mineral- und organischen Substanzen in den Blättern und geschälten Samen von *Aesculus Hippocastanum* während ihrer Entwicklung. Beitr. z. Biol. d. Pflanzen **20**, 179–197 (1932).
8) Ebermayer, E., Die gesammte Lehre der Waldstreu. Berlin (1876).
9) Échevin, R., L'azote, le phosphore et le soufre chez les plantes ligneuses à feuilles caduques. Rev. gén. bot. **43**, 517–677 (1931).
10) Échevin, R., Sur quelques formes biologiques du phosphore des feuilles chez les arbres. Rev. gén. botan. **48**, 505–517 (1936).
11) Euler, H. v. and Schmidt, G., Über Nucleoproteide der Fisch-Testikel. Z. physiol. Chem. **225**, 92–102 (1934).
12) Fries, N., The effect of certain nucleic acid constituents on the growth of some higher fungi. Nature (in press) (1951).
13) Fries, N. and Björkman, U., Microbiological determination of adenine and guanine. Physiologia Plantarum **2**, 212–215 (1949).
14) Fries, N. and Forsman, B., Quantitative determination of certain nucleic acid derivatives in pea root exudate. Physiologia Plantarum **4**, 410–420 (1951).
15) Hammarsten, E., On the extraction of nucleotides from cells. Acta med. scand., Suppl. **196**, 634–645 (1947).
16) Jones, R. F. and Baker, H. G., The effect of some purines and related compounds on the seedling growth of *Avena sativa* L. Ann. Botany, N.S. **7**, 379–390 (1943).
17) Kaila, A., Viljelysmaan orgaanisesta fosforista (On the organic phosphorus in cultivated soils). Thsesis, Helsinki (1948).
18) Knudsen, F. and Mauritz-Hansson, H., Om produktionen av lövförna och dennas sammansättning i ett mellansvenskt björkbestånd. Svenska skogsvårdsfören. tidskr. **37**, 339–347 (1939).
19) Koch, A. and Oelsner, A., Über Nukleoproteid spaltende Bakterien und ihre Bedeutung für die Erschliessung des Phosphorkapitals im Ackerboden. Biochem. Z. **134**, 76–96 (1923).
20) Kossel, A., Zur Chemie des Zellkerns. Z. physiol. Chem. **7**, 7–22 (1882).
21) Kruyt, W. and Veldstra, H., Plant growth regulators. XIII. Leaf growth factors. Koninkl. Akad. Wetenschappen, Amsterdam, Proc. **50**, 1142–1149, 1317–1323 (1947).
22) Levene, P. A. and Bass, L. W., The nucleic acids. Chemical Catalog Company, New York (1931).
23) Lindquist, B., Dalby Söderskog. Acta phytogeogr. suecica No. 10. Stockholm (1938).
24) Mejbaum, V., Über die Bestimmung kleiner Pentosemengen, insbesondere in Derivaten der Adenylsäure. Z. physiol. Chem. **258**, 117–120 (1939).
25) Melin, E., Der Einfluss von Waldstreuextrakten auf das Wachstum von Boden-

pilzen, mit besonderer Berücksichtigung der Wurzelpilze von Bäumen. Symb. bot. upsal. **8**, No. 3. Uppsala (1946).

26) Michael, G. and Heidecker, L., Ein weiterer Beitrag zur Frage der Veränderlichkeit der Phosphorsäurefraktionen im Spinat in Abhängigkeit von verschiedener Phosphorsäuredüngung. Bodenkunde u. Pflanzenernähr. **17**, 358–372 (1940).

27) Mockeridge, F. A., The occurrence and nature of the plant growth-promoting substances in various organic manurial composts. Biochem. J. **14**, 432–450(1920).

28) Norkrans, B., Studies in growth and cellulolytic enzymes of *Tricholoma*. With special reference to mycorrhiza formation. Symb. bot. upsal. **11**, No. 1. Uppsala (1950).

29) Olsen, C., The mineral, nitrogen and sugar content of beech leaves and beech leaf sap at various times. Compt. rend. Lab. Carlsberg, Série chimique **26**, 197–230 (1948).

30) Ramann, E., Wandern die Nährstoffe beim Absterben der Blätter? Z. f. Forst- u. Jagdwesen **30**, 157–176 (1898).

31) Romell, L.-G., Den nordiska blåbärsgranskogens produktion av ris, mossa och förna. Svensk Botan. tid. **33**, 366–382 (1939).

32) Sampson, A. W. and Samisch, R., Growth and seasonal changes in composition of oak leaves. Plant Physiol. **10**, 739–751 (1935).

33) Schmidt, G. and Thannhauser, S. J., A method for the determination of desoxyribonucleic acid, ribonucleic acid, and phosphoproteins in animal tissues. J. Biol. Chem. **161**, 63–89 (1945).

34) Schneider, W. C., Phosphorus compounds in animal tissues. I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. J. Biol. Chem. **161**, 293–303 (1945).

35) Schreiner, O. and Shorey, E. C., Chemical nature of soil organic matter. U.S. Dept. Agr., Bureau of soils, Bull. No. 74 (1910).

36) Schreiner, O. and Skinner, J. J., Nitrogenous soil constituents and their bearing on soil fertility. U.S. Dept. Agr., Bureau of soils, Bull. No. 87 (1912).

37) Schulze, B. and Schütz, J., Die Stoffwandlungen in den Laubblättern des Baumes, insbesondere in ihren Beziehungen zum herbstlichen Blattfall. Landw. Vers. Stat. **71**, 299–352 (1909).

38) Shorey, E. C., Some organic soil constituents. U.S. Dept. Agr., Bureau of soils, Bull. No. 88 (1913).

39) Staffel, E., Über die Menge der unorganischen Bestandteile in den gleichen Organen ein und derselben Pflanze (der Rosskastanie und des Walnussbaumes) während verschiedener Vegetations-perioden. Arch. Pharm., 2e Serie **64**, 1–129 (1850).

40) Swart, N., Die Stoffwanderung in ablebenden Blättern. Jena, 1914.

41) Teorell, T., Spektrophotometrische Mikrobestimmung des Phosphors. Biochem. Z. **230**, 1–13 (1931).

42) Tonzig, S., Osservationi sul ricambio purinico dei nucleo-proteidi nelle piante. Annali di Botanica **20**, 83–105 (1933).

43) Winterstein, A. and Somló, F., Purine, Pyrimidine und verwandte Verbindungen. Handb. d. Pflanzenanalyse herausgeg. v. G. Klein, Spezielle Analyse **3**, 362–405 (1933).

44) Williams, H. F., The estimation of nucleic-acid phosphorus in plant material. Australian J. Exp. Biol. Med. Sci. **23**, 213–219 (1945).

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CONTRIBUTION TO THE CHEMICAL AND BACTERIOLOGICAL OXIDATION OF PYRITE IN SOIL

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INTRODUCTION

Considerable amounts of sulphides are often formed in soils under anaerobic conditions, especially when these soils are rich in organic compounds and sulphates. This process results from the enzymatic action of sulphate-reducing micro-organisms. When such soils later become exposed to air, the sulphides are oxidised and much sulphuric acid is formed, resulting in high acidity if the soils lack carbonates. Van Bemmelen[1]) already has shown that both monosulphides and polysulphides are thus transformed to sulphuric acid. In recent publications van der Spek[10]) and Harmsen, Quispel and Otzen[2]) support the opinion of van Bemmelen. Oxidation of iron-monosulphide and the part bacteria take in this process has been fairly well studied (Verhoop[11]), but scarcely anything is known about the oxidation of the equally important pyrite. Rudolfs[8]) suggested that this oxidation is accelerated by bacteria, and that elementary sulphur acts as catalyst. Jensen[3]) also demonstrated the stimulation of this oxidation by bacteria (presumably *Thiobacillus thiooxydans*), though the process also proved to be possible under sterile conditions. He found low pH values to be very favourable. Another important contribution was the work of McIntire, Shaw and Young[5]) They determined the formation of sulphates from added pyrite in the effluent of lysimeters with soil. Their conclusion was identical with that of Jensen: pyrite is oxidised equally well with or without the aid of bacteria, but the latter accelerate the process significantly.

Widespread occurrence of acid soils in the newly reclaimed marshlands of the Zuidersea, as a result of oxidation of pyrite, made a new investigation necessary. This work was started in 1933 by Verweel and was later continued by Matla (unpublished data). These preliminary investigations have confirmed older findings, that pyrite could be oxidised even at low temperatures in sterile media, but certain bacteria greatly accelerated this process. These investigators also found, in agreement with Jensen [3]) that the process was stimulated by low pH values. In recent years this investigation was continued by the present authors. At first our efforts to repeat the experiments of Verweel and Matla had no success: when highly purified pyrite was used, no oxidation of the latter could be achieved either with or without the specific sulphur oxidising bacteria. Only in crude pyrite preparations was sulphuric acid formed. It seamed therefore likely that either this oxidation requires some still unknown conditions, or that the oxidation observed in the older investigations was exclusively due to some sulphur containing impurities of the pyrite used.

It was the purpose of the present investigation to check the older reports and to clarify this controversy. A series of new experiments was therefore started, using highly purified pyrite as well as crude pyrite containing substrates, whose composition was analysed by the technique of Smittenberg *et al.* [9]), as accurately as possible.

EXPERIMENTAL

In all these experiments 400 mg of pyrite was suspended in 40 ml of the medium in Erlenmeyer flasks of 100 ml. Different pyrites were prepared from the crude commercial pryite by the following treatments: The original pyrite was first ground in a laboratory mill through a sieve with meshes of 0.25 mm diameter, then this powder was further disintegrated in a ball-mill and the coarser particles were removed by gentle washing. The resulting powders, of different grades of fineness, were then submitted to three consecutive extractions by boiling with concentrated hydrochloric acid for three hours. As the powders treated in this way still proved to contain much elementary sulphur, some of them were extracted further (three times) with carbon-tetrachloride for a day in a Soxhlet apparatus. The following products were used for experiments:

A — Crude pyrite, ground in a Whiley-type laboratory mill.
B — Product *A* extracted with HCl.
C — Product *B* extracted with CCl_4.
D — Product *A* ground in a ball-mill.
E — Product *D* extracted with HCl.
F — Product *E* extracted with CCl_4.

The original crude pyrite did not contain monosulphides, as it released no hydrogen sulphide when treated with dilute hydrochloric acid. But concentrated hydrochloric acid released small amounts of hydrogen sulphide readily. This reaction could not be due to the action of hydrochloric acid on the pyrite itself, since the latter is resistant to concentrated hydrochloric acid (Smittenberg *et al.* [9]). Consequently other, less resistant sulphur compounds, presumably polysulphides were present in the crude pyrite (0.038%). Of course products *B*, *C*, *E* and *F* were free of these impurities. The content of elementary sulphur proved to be about 0.25% in products *A*, *B*, *D* and *E*, while it was absent in *C* and *F*. The presence of other impurities, which could give sulphates when the pyrite was oxidised, could not be demonstrated.

As nutrient media for suspending the pyrite powders, the following solutions were used:

I – $(NH_4)_2SO_4$, 0.2 g; $CaCl_2$, 0.25 g; $MgSO_4 . 7H_2O$, 0.5 g; K_2HPO_4, 3.0 g; $FeSO_4$, 0.01 g in 1000 ml of tap-water; pH adjusted to 6.5.
II – The same as I, but acidified with H_2SO_4 to pH of 4.0.
III – $(NH_4)_2SO_4$, 0.4 g; $CaCl_2$, 0.25 g; $MgSO_4.7H_2O$, 0.25 g; K_2HPO_4, 4.0 g; $FeSO_4$, 0.01 g; $NaHCO_3$, 5.0 g (separately sterilised), in 1000 ml of tap-water; pH was adjusted to 7.6.
IV – The same as III, but without $NaHCO_3$; pH adjusted to 5.8.
V – The same as IV, but K_2HPO_4 replaced by KH_2PO_4; the pH adjusted to 3.9.
VI – The same as IV, but acidified with H_2SO_4 to pH of 3.9.

Dispersing pyrite powder in the solutions was not difficult when the relatively coarse powders *A*, *B* or *C* were used, but the very fine, ball-mill ground, powders *D*, *E* or *F* required special care to avoid the formation of clusters and larger conglomerates. The powder was first carefully mixed in a mortar with a small amount of the solution to form a thick suspension, then the coarsest particles were

removed by precipitation after shaking, and finally the suspension was diluted with nutrient solution to the required concentration.

Generally these suspensions were sterilised by autoclaving for half an hour at 120°C. This sometimes shifted the pH to the acid side; it was then restored with NaOH. All experimental series were performed with several replicates. The temperature of incubation was held either at 30 or at 60°C.

Oxidation of the pyrite could be followed continuously by determining the pH during incubation. With longer intervals, increases of soluble sulphate and iron were determined to measure the amount of pyrite oxidised. Herefore both sulphate and iron, soluble in water and soluble in concentrated hydrochloric acid, were determined. For iron the titrimetric procedure of Tread-well[13]) was followed. In the earlier phases of the investigation, the gravimetric method of Treadwell[13]) was used for determining sulphate, but later the faster and more adequate titrimetric method of Raschig, as modified by Pieters[7]) or by Jones and Wood[4]), was followed.

RESULTS

Series I

In the first series the influence of microbes on oxidation of pyrite had to be checked. Therefore some flasks were inoculated with a suspension of a soil that showed a vigorous formation of sulphuric acid from pyrite. Other duplicate flasks remained uninoculated and served as blanks. Some of the replicate flasks were aerated with sterile air, but generally the flasks were kept as still cultures. However the shallowness of the liquid layer always ensured a moderate aerobiosis. The composition of the cultures was also varied according to the different grades of fineness and purification of the pyrite (*A*, *B*, *C* or *D*) or according to the pH of the medium by using alternatively the solutions I or II. Most of the flasks were incubated at 30°C. Some replicates were kept at 60°C, at which temperature no development of microbes could be expected, while the contingent chemical oxidation should be accelerated. All cultures of this series were analysed before incubation and after 2.5 and 5 months of incubation. The results are summarised in Table I.

Only a few of the cultures were started in sufficiently high numbers of flasks to allow duplicate determinations, but these

TABLE I

Soluble iron and sulphate at the beginning of the experiment and after incubation, expressed as milligramatoms of Fe and S per flask. The figures at the beginning refer to the amount of soluble Fe and SO_4, the figures after 2 and a half and after 5 months of incubation respectively refer to the increase in Fe and SO_4 as compared with the amount at the beginning.

No. of the series	pyrite	medium	inoculation	aeration	temperature of incubation	At the beginning					After 2½ months of incubation					After 5 months of incubation					
						Soluble in water			Soluble in HCl		Soluble in water (increase)			Soluble in HCl (increase)		Soluble in water (increase)			Soluble in HCl (increase)		
						pH	Fe	SO_4	Fe	SO_4	pH	Fe	SO_4	Fe	SO_4	pH	Fe	SO_4	Fe	SO_4	
1	A	I	+	—	30	6.7	0.00	0.26	0.10	0.02	3.1	0.07	0.20	0.13	0.02	—	0.83	1.80	—	—	
2	A	I	—	—	30	6.7	0.00	0.26	0.10	0.02	5.1	0.00	0.00	0.08	0.01	—	0.00	0.02	—	—	
3	A	II	+	—	30	4.6	—	0.28	—	—	—	—	—	—	—	2.1	1.11	2.45	—	—	
3a	A	II	+	—	30	4.6	—	—	—	—	—	—	—	—	—	—	1.13	2.34	—	—	duplo
4	A	II	—	—	30	4.6	—	0.28	—	—	—	—	—	—	—	3.3	0.01	0.05	—	—	
5	B	I	+	—	30	6.2	0.00	0.24	0.03	0.00	1.8	0.79	1.71	0.00	0.00						
6	B	I	—	—	30	6.2	0.00	0.24	0.03	0.00	2.9	0.05	0.22	0.00	0.00						
7	B	II	+	—	30	3.1	—	0.29	—	—	—	—	—	—	—	1.8	1.49	3.14	—	0.00	
8	B	II	—	—	30	3.1	—	0.29	—	—	—	—	—	—	—	2.7	0.01	0.04	0.04	0.00	
9	C	I	+	—	30	6.3	0.00	0.15	0.03	0.00	3.0	0.00	0.09	0.06	0.01						
10	C	I	—	—	30	6.3	0.00	0.15	0.03	0.00	3.6	0.00	0.07	0.06	0.01						
11	C	I	—	—	60	6.3	0.00	0.15	0.03	0.00	1.6	0.56	0.96	0.00	0.02						
12	C	I	+	+	30	6.3	0.00	0.15	0.03	0.00	5.1	0.00	0.08	0.05	0.02						
13	C	I	—	+	30	6.3	0.00	0.15	0.03	0.00	3.4	0.00	0.07	0.06	0.02						
14	C	II	+	—	30	3.5	—	0.20	—	—	1.9	0.76	1.65	—	—						
15	C	II	—	—	30	3.5	—	0.20	—	—	2.7	0.05	0.29	—							
16	D	I	+	—	30	6.9	0.08	0.60	0.18	0.12	1.7	0.81	2.29	0.47	0.37	—	1.05	3.22	0.62	0.53	
16a	D	I	+	—	30	6.9	—	—	—	—	—	—	—	—	—	—	1.96	4.60	0.57	0.39	duplo
17	D	I	—	—	30	6.9	0.08	0.60	0.18	0.12	2.2	0.24	0.63	0.13	0.11						
18	D	I	+	+	30	6.9	0.08	0.60	0.18	0.12	1.4	2.13	5.10	—	0.43	—	—				
19	D	I	—	+	30	6.9	0.08	0.60	0.18	0.12	2.2	0.25	0.53		—	—					
20	D	II	+	—	30	4.2	—	0.60	—	—	—	—	—	—	—	1.6	1.60	4.01	—	—	
21	D	II	—		30	4.2	—	0.60	—	—		—	—	—	—	2.1	0.42	0.92	—	—	

examples showed that the reliability of the method applied was not very high — presumably because of variation in the initial pH of individual flasks — though sufficient to justify some general conclusions:

1. The pyrite was apparently significantly oxidised. In most flasks the amount of iron and sulphate dissolved was much too high to be explained solely as oxidation of impurities. In some flasks of the series *D* II, incubated for more than 5 months, even the pyrite was all destroyed at the end of 6 months of incubation (not included in the table).

2. The sterile flasks, incubated at 60°C (11), those incubated at 30°C and aerated (19) and those with the very fine and pure pyrite (21) showed such a vigorous oxidation, that it is beyond any doubt that pyrite under favourable conditions can readily be oxidised in sterile media. These three flasks remained sterile throughout the incubation.

3. Oxidation, however, was often significantly stimulated by bacterial action (compare series 1 with 2, 3 with 4, 5 with 6, 7 with 8, 14 with 15, 16 with 17, 18 with 19 and 20 with 21).

4. Other factors favouring oxidation were: low initial pH (compare 1 with 3 or 12 with 14), high temperature (compare 10 with 11) and aeration (compare 16 with 18). It is surprising that the last factor became apparent only in the inoculated series 16 and 18, not in the comparable sterile set 17 and 19.

5. No clear relation was found between purity of pyrite and its oxidisability; yet elementary sulphur (extractable with CCl_4) seems to have stimulated oxidation (compare 5 with 9).

6. The iron and sulphate liberated by oxidation proved in most cases to be soluble in water. However, when oxidation was very far advanced (16 and 18), part of the oxidation product had to be extracted with concentrated hydrochloric acid. In such flasks a yellow precipitate of basic ferric sulphate was formed.

Series II

The first series gave clear-cut answers to some questions concerning the oxidation of pyrite, but other problems were left unsolved and new questions were raised. One of the unsolved problems is the discrepancy between the striking resistance against oxidation reported in some of the older investigations, and the relatively fast oxidation

achieved in some of the media of series I. Another factor requiring further study is the effect of impurities on the rate of oxidation. Since the figures in Table I showed that elementary sulphur is presumably the most important impurity in pyrite, in the next experiments pure elementary sulphur was added to purified pyrites. However, during the experiment this sulphur can be expected to be quickly transferred to sulphates; therefore in medium VI sulphate ions were added directly. In this second series only the most purified pyrites *C* and *F* were used, suspended in media III, IV, V and VI. Otherwise the experimental conditions were identical with those of series I. The analytical data at the beginning and after 3 and 5 months of incubation are summarised in Table II.

These figures confirmed the results of series I that:

1. The oxidation is stimulated by low pH levels (compare 9 and 12 with 16); at alcaline pH even at 60°C scarcely any oxidation was visible after 5 months of incubation.

2. The presence of sulphur-oxidising bacteria significantly accelerated the oxidation (compare 3 with 4, 7 with 8, 14 with 15 and 16 with 17), and

3. Higher temperatures also stimulate the oxidation (compare 10 with 11 and 17 with 18).

In addition, stimulation of the oxidation of pyrite by elementary sulphur was clearly demonstrated (compare 3 with 1 and 14 with 12 or 15 with 13). Of course, in flasks with added sulphur (3, 4, 14 and 15), formation of sulphates and the shift of the reaction to the acid are not indications of pyrite oxidation. But the formation of soluble iron compounds shows that pyrite was attacked. Comparison of flasks 3 and 14 with flasks 7 and 16 shows that acceleration of the oxidation of pyrite was due to high acidity produced by rapid oxidation of the sulphur to H_2SO_4, since acidification of the medium with sulphuric acid or KH_2PO_4 (7 and 16) had a similar effect.

But the most remarkable phenomenon in this second series is the much slower progress of the oxidation than in the first series. The exclusive use of highly purified pyrites (*C* or *F*) cannot be the reason since in the first series pyrite *C* showed vigorous oxidation, while some flasks in which the unpurified pyrite *D* was used (not included in the Table), now showed the same very slow oxidation. It must also be pointed out that, while in the first series both iron and sulphur from the pyrite were transformed to water soluble compounds, now

TABLE II

Soluble iron and sulphate at the beginning of the experiment and after incubation, expressed as milligramatoms of Fe and S per flask. The figures at the beginning refer to the amount of soluble Fe and SO_4, the figures after 3 and after 5 months of incubation respectively refer to the increase in Fe and SO_4 as compared with the amount at the beginning.

No. of the series	pyrite	medium	sulphur 5%	inoculation	temperature of incubation	At the beginning			After 3 months of incubation					After 5 months of incubation				
						pH	Soluble in water		pH	Soluble in water (increase)		Soluble in HCl (increase)		pH	Soluble in water (increase)		Soluble in HCl (increase)	
							Fe	SO_4		Fe	SO_4	Fe	SO_4		Fe	SO_4	Fe	SO_4
1	C	IV	—	+	30	6.8	0.00	0.35	6.0	0.00	0.12	—	—	5.9	—	0.14	—	—
2	C	IV	—	—	30	6.8	0.00	0.35	6.0	0.00	0.09	—	—	6.4	—	0.10	—	—
3	C	IV	+	+	30	6.8	0.00	0.41	6.0	0.00	0.21	—	—	1.6	0.04	1.22	0.24	0.05
4	C	IV	+	—	30	6.8	0.00	0.41	6.2	0.00	0.22	—	—	5.8	—	0.26	—	—
5	C	V	—	+	30	3.9	0.00	0.27	2.6	0.00	0.05	—	—	2.3	—	—	—	—
6	C	V	—	—	30	3.9	0.00	0.27	2.9	0.00	0.00	—	—	2.9	0.00	0.02	—	—
7	C	VI	—	+	30	3.9	0.00	0.59	2.2	traces	0.29	—	—	2.0	0.04	0.69	—	—
8	C	VI	—	—	30	3.9	0.00	0.59	2.9	0.00	0.05	—	—	2.9	—	0.07	—	—
9	F	III	—	+	30	7.6	0.00	0.27	7.5	0.00	0.11	—	—	7.3	—	0.12	—	—
10	F	III	—	—	30	7.6	0.00	0.27	7.4	0.00	0.14	—	—	7.2	—	0.19	—	—
11	F	III	—	—	60	7.6	0.00	0.27	6.2	0.00	0.14	—	—	6.0	—	0.30	—	—
12	F	IV	—	+	30	6.8	0.00	0.42	3.5	0.00	0.18	—	—	2.7	—	0.30	—	—
13	F	IV	—	—	30	6.8	0.00	0.42	4.9	0.00	0.21	—	—	3.6	—	0.27	—	—
14	F	IV	+	+	30	6.7	0.00	0.42	1.7	0.10	1.47	0.21	0.09	1.6	0.18	1.58	0.35	0.11
15	F	IV	+	—	30	6.7	0.00	0.42	3.2	0.00	0.30	—	—	2.7	—	0.39	—	—
16	F	V	—	+	30	3.1	0.00	0.27	1.7	0.07	0.92	0.27	0.08	1.5	0.13	1.32	—	—
17	F	V	—	±	30	3.1	0.00	0.27	2.3	0.00	0.26	—	—	2.2	—	0.25	—	—
18	F	V		—	60	3.1	0.00	0.27	2.2	0.00	0.43	0.12	0.02	1.6	0.04	1.70	—	

only the sulphate was found in the aqueous extract; iron, on the contrary, could be demonstrated only in insignificant amounts. The bulk of the oxidised iron was HCl-extractable. It was also observed that in the first series a yellow precipitate of iron sulphates was formed, while in this series a pale whitish precipitate of iron phosphate accumulated.

A satisfactory explanation for this fundamentally different course of the oxidation in the two series has not yet been found. The only general difference between the two media used was the slightly higher concentration of phosphate in the second series (0.4 instead of 0.3‰). Such a difference scarcely seemed significant. Yet the uniform formation of ferric phosphate instead of ferric sulphate in all flasks with retarded oxidation suggests a relation between the two phenomena. As a hypothesis, therefore, it is suggested that phosphate ions have a tendency to interfere with the oxidation of pyrite. An attempt was made to check this hypothesis in the following experiment: The finely ground and purified pyrite *F* was dispersed in different buffer solutions. One was phthalate buffer (50 ml of 0.2 m primary potassium phthalate + 43 ml of 0.2 m NaOH, made to 200 ml) with a pH of 5.8. Another, with the same pH, was a phosphate buffer (50 ml of 0.2 m KH_2PO_4 + 3.72 ml of 0.2 m NaOH, made to 200 ml). The third was a similar phosphate buffer but with a pH of 7.8 (50 ml of 0.2 m KH_2PO_4 + 45.2 ml of 0.2 m NaOH, made to 200 ml). The fourth, finally, with the same pH of 7.8, was a borate buffer (50 ml of 0.2 m H_3BO_3, 0.2 m KCl + 2.61 ml of 0.2 m NaOH, made to 200 ml). The flasks with these suspensions were incubated without inoculation at 55°C. Each of the four different suspensions was dispensed over 6 flasks; at intervals of one to two weeks water soluble sulphur was determined in one of the flasks of each series. Variability between individual flasks in one series was of course higher than between samples from one larger batch, (which may be the explanation of the peak after 15 days) but the analyses agreed sufficiently to be used for graphical expression in the accompanying figure.

This experiment fully confirmed the above hypothesis; at both pH levels in the phosphate buffer oxidation was significantly lower than in other buffers. Just how the small difference in phosphate concentration between the media of the first and of the second series of experiments could bring about such a striking difference in pyrite

oxidation, remains unexplained. It may be that a critical phosphate concentration decides which course of oxidation will be followed; to ferric phosphate or to ferric sulphate. This and several other problems are still unsolved and require further investigation, but fundamentally the oxidation of pyrite can be considered sufficiently studied to allow a general outline of this process.

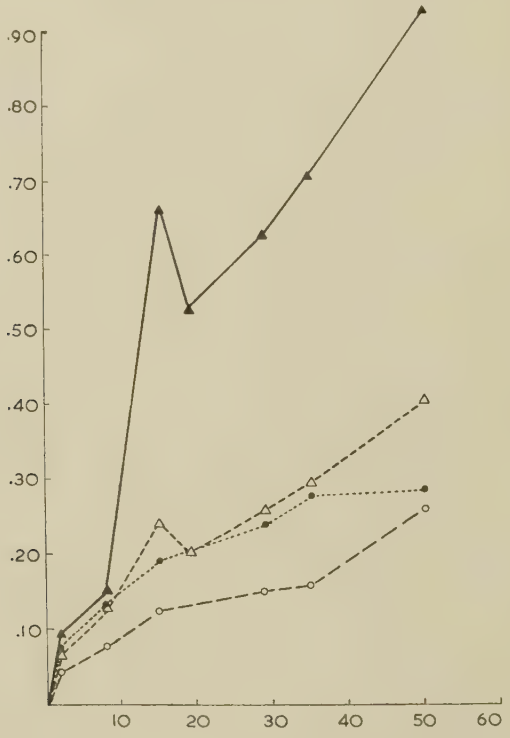

Fig. 1. Oxidation of pyrite at 55°C. Abscis; time in days. Ordinate; increase of sulfate, soluble in water in milligramatoms per flask.
▲———▲ in phthalate buffer pH 5.8
●·····● in borate buffer pH 7.8
△- - - - -△ in phosphate buffer pH 5.8
○ — — ○ in phosphate buffer pH 7.8

DISCUSSION

V e r h o o p [14]) has shown that oxidation of iron monosulphide proceeds through two stages: The first step is oxidation of Fe to Fe_2O_3. Microbes take only an insignificant part in this process. The

second step is oxidation of the released elementary sulphur to sulphates. This process proceeds slowly at low temperatures without the aid of bacteria. Although the method used by Verhoop to separate the two steps cannot be applied to the much slower oxidation of pyrite, it may be presumed that pyrite oxidation will follow the same pattern. Therefore we can assume that oxidation of pyrite also starts with oxidation of the iron. It follows that any factor that reduces the solubility of iron must suppress the progress of this process. Phosphate ions (formation of the very insoluble Fe_3PO_4) or hydroxyl ions (high pH) may become active as such factors. This pronounced dependence of the oxidation of pyrite upon the solubility of iron explains the fact that in the earlier work in this laboratory no oxidation of pyrite could be shown, whereas in the present investigation this oxidation undoubtedly occurred. The media in the earlier experiments were less favourable (too high pH and too much phosphate).

Through oxidation of the iron, sulphur is released either as elementary sulphur or as sulphide ions. Thus endothermic substances are formed, which can be used by the sulphur-oxidising microbes. The oxidation of sulphur may be considered a final reaction, from which the microbes derive free energy, contrary to the degradation of many other insoluble endothermic substances (polysaccharides, fats, etc.). In all those reactions the solubilisation of the original substance is only the first step, while liberation of the bulk of the energy is achieved by later phases of the transformation of the first soluble disintegration products. Therefore the hydrolysis of polysaccharides and most other insoluble substances can be an exoenzymatic process, but the oxidation of elementary sulphur can only be understood by assuming that it is an endoenzymatic reaction. Therefore sulphur oxidation starts only when the pyrite is disintegrated by oxidation of the iron. Part of the released sulphur may then be absorbed directly by the bacterial cells as sulphide ions, but the bulk of the sulphur first accumulates in the elementary form. The mechanism of the penetration of this elementary sulphur into cells is still obscure. Vogler and Umbreit[15]) and Umbreit, Vogel and Vogler[16]) suggest solution of the elementary sulphur in the fat droplets near the poles of the cells. These fat droplets are characteristic for the most active sulphur-oxidising microbes — *Thiobacillus thiooxidans*.

Although in the present investigation this hypothesis of Umbreit was not checked, the role of the specific sulphur-oxidising microbes for oxidation of pyrite, as already claimed by Jensen [3] and by McIntire, Shaw and Young [5] could be confirmed. Using the isolation methods described by Starkey [11] [12], sulphur-oxidising bacteria could be readily isolated from soil. In ordinary soils only strains of *Thiobacillus thioparus* could be demonstrated in this way, but in the pyrite-containing soils of the newly reclaimed marine deposits, still incompletely oxidised and weathered, *Thiobacillus thiooxidans* predominated. This organism was also found as nearly pure culture in those flasks of the foregoing experiments that showed a vigorous oxidation of pyrite. The isolated cultures could easily be purified and identified. Thus it was clearly demonstrated that the species most important for oxidation of pyrite is *Thiobacillus thiooxidans*, the same organism that is also responsible for oxidation of monosulphides and other sulphur compounds. It is possible that, in the initial stages of the oxidation, other species (*Th. thioparus*) are active, but they are soon crowded out by *Th. thiooxidans* in the older fermentations, when high concentrations of sulphates are formed, and even more so in acidified soils with insufficient amounts of carbonates.

The results of the above experiments and contemplations justify the general conclusion that it is not necessary to design complicated reaction schemes to explain the oxidation of pyrite, as Ohle [6] did. The course of the oxidative degradation of pyrite may be assumed to be rather simple: The ferrous ions on the surface of a pyrite crystal are oxidised to Fe_2O_3, when not counteracted by high pH levels or high concentration of phosphate ions. Thus the sulphur of the molecules in the surface layer becomes released or temporarily forms (with the intact pyritemolecules of the deeper layers) labile polysulphides with a higher ratio of sulphur to iron than in pyrite. Finally the excess sulphur becomes oxidised by microbes and forms sulphates. The pH then decreases, which again accelerates the oxidation of the iron, therefore the whole process, once started, is speeded up more and more.

SUMMARY

1. The rate of pyrite oxidation and the influence of microbes on this process were studied, since this oxidation proved to be one of the most

important phenomena in the process of weathering and aging of newly reclaimed marine soils.

2. Pyrite oxidation does not depend on the activity of microbes, but is significantly stimulated by the metabolism of the specific sulphur-oxidising bacteria. The most important species proved to be *Thiobacillus thiooxidans*, the same organism that takes part in the oxidation of monosulphides, thiosulphates, etc.

3. Factors depressing the solubility of iron (high pH level or high concentration of phosphate ions) retard the oxidation of pyrite.

4. It is probable that the oxidative disintegration of pyrite starts with the oxidation of the ferrous ions on the surface of the crystals, releasing the sulphur. The latter is then oxidised primarily by the specific sulphur-oxidising microbes and transformed to sulphates.

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REFERENCES

1) Bemmelen, J. M. van, Bijdrage tot de kennis van den alluvialen bodem in Nederland. III. De samenstelling en vorming van de zure gronden in het Nederlandsch Alluvium. Verhandel. Acad. Wetenschappen, Amsterdam **25** (1886).
2) Harmsen, G. W., Quispel, A. and Otzen, D., Observations on the formation and oxidation of pyrite in the soil. Plant and Soil, in press.
3) Jensen, H. L., Vorkommen von *Thiobacillus thiooxidans* in daenischen Böden. Zentr. Bakt., Parsitenk., II **72**, 242 (1927).
4) Jones, D. G. and Wood, C. E., J. Inst. Petroleum Techn. **18**, 817 (1932).
5) McIntire, W. H., Shaw, W. M. and Young, J. B., The oxidation of pyrite and sulphur as influenced by lime and magnesia — a 12 year lysimeter study. Soil Sci **30**, 443 (1930).
6) Ohle, W., Der schwefelsaure Tonteich bei Reinbek. Arch. Hydrobiol. **30**, 604 (1936).
7) Pieters, M. A. J., Sulphaatbepaling. Chem. Weekblad **39**, 21 (1942).
8) Rudolfs, W., Oxidation of iron-pyrites by sulphur-oxidising organisms. Soil Sci. **14**, 135 (1922).
9) Smittenberg, J., Harmsen, G. W., Quispel, A. and Otzen, D., Rapid methods for determining different types of sulphur compounds in soil. Plant and Soil **3**, 353—360 (1951).
10) Spek, J. van der, Bijdrage tot de kennis van de zure gronden in het Nederlandsch Alluvium. Verslag. Landb. Onderzoek No. 40B (1934).
11) Starkey, R. L., Cultivation of organisms concerned in the oxidation of thiosulphate. J. Bact. **28**, 365 (1934).
12) Starkey, R. L., Isolation of some bacteria, which oxidise thiosulphate. Soil Sci. **39**, 197 (1935).
13) Treadwell, F. P., Kurzes Lehrbuch der analytischen Chemie I (1908).
14) Verhoop, A., Chemische en microbiologische omzettingen van ijzersulphiden in den bodem. Thesis, Leiden-Wageningen (1940).
15) Vogler, K. G. and Umbreit, W. W., The necessity for direct contact in sulphur oxidation by *Thiobacillus thiooxydans*. Soil Sci. **51**, 331 (1941).
16) Umbreit, W. W., Vogel, H. R. and Vogler, K. G., The significance of fat in sulphur oxidation by *Thiobacillus thiooxydans*. J. Bact. **43**, 141 (1942).

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SYMBIOTIC ADAPTATION IN LOCAL STRAINS OF RED CLOVER AND NODULE BACTERIA

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The hereditary basis of symbiotic nitrogen fixation has been little investigated. Studies on species and varietal relationships [12] [2] and hybridization experiments in red clover [6] suggest that the symbiotic phenotype cannot be adequately described in terms of any property or combination of properties of one of the symbionts only, but is an expression of the interaction of hereditarily determined plant and bacterial components. On the plant side (in red clover) the genetic determinants concerned in nitrogen fixation have been shown in some instances to be single factors, reacting specifically with bacterial strains, and in others to have a complex mode of inheritance when no specific interrelations seem to be involved [8]. Other inherent host factors influence the symbiosis independently of the amount of nitrogen fixed. On the bacterial side, genetic analysis of strain differences is not possible; such differences are however stable and transmissible. Bacterial strains have also been shown in the laboratory to be subject to mutative changes [1] [5] some of which represent adaptation to simple and specific genotypic differences in the host.

Adaptation in the field may also be effected by mutual adjustment involving genetic components in each organism. A number of species of *Trifolium* [11] [3] and *Medicago* [3] and *Melilotus* [12] are known to give distinct effectivity patterns with bacterial strains isolated from each, but there do not appear to be any records of any specific relation between plant and strain within a botanical species.

The object of this study is to examine local strains of red clover for evidence of adaptation to local strains of nodule bacteria although no attempt will be made to analyse genetically any differences which may appear.

MATERIAL AND METHODS

Local strains of red clover are well established and clearly defined in Sweden, and through the kindness of Dr. Bjälfve and Dr. Julén samples of six Swedish strains have been obtained together with isolates of nodule bacteria made from them while growing in their own localities. In addition, three British samples of clover were obtained with their homologous isolates with the assistance and advice of Dr. Watkin Williams. These are listed below with their place of origin and approximate age as recognised strains. Information on the Swedish material is given by Åkerberg and Julén[1]) and in personal communication from Dr. Julén.

	English Strains	*Locality*	*Age*
A	Cornish marl	E. Devon	?
B	Cornish marl	S. Devon	?
C	Late-flowering Montgomeryshire red	Montgomeryshire	?
	Swedish Strains		
P	Merkur (Spannarp)	A strain bred at Svalöf (Skäne)	> 50 years
Q	Hassle Säby	Västergötland	> 50 years
R	Sörby Okna	Östergötland	49 ,,
S	Ultuna	Uppsala	44 ,,
T	Offer	Västernorrland	> 25 ,,
U	Kusträsk	Norrbotten	40 ,,

The location of the farms of origin of the Swedish seed are shown on the accompanying map (Fig. 1).

In the following tables the local strains of clover will be distinguished by the capital letters *A*, *B*, ... *U* and the strains of bacteria isolated from the localities in which they are grown by the letters a_1, a_2, ... u_4. From four to twelve isolates were made from each locality; these consisted in the Montgomeryshire material of separate isolates from three nodules on each of four plants and in the remainder of paired isolates from each plant (from 1 plant of *U* and from 2–4 plants of each of the remainder).

The bacteria isolated from the nodules were maintained on a yeast mannitol medium. Each inoculation in the principal experiment was made from a fresh culture after plating.

The seed of each local strain was carefully selected for size and freedom from injury to testa before sterilising with concentrated H_2SO_4 for 30 minutes and was then washed frequently with sterile water. Each seed was

Fig. 1. Map of Sweden, showing Stations of the Swedish Seed Association and Places of Origin of the Local Strains of Clover.

planted singly in 6″ × ¾″ test tube containing 8 ccs of a sloped nitrogen free agar medium [10]) and 2 ccs of liquid culture medium. Only normal seedlings uncontaminated with fungus were retained which were then inoculated at 2 weeks with a loopful of a suspension of the bacteria in saline. Each of the 52 isolates was inoculated to at least four plant cultures of clover from its own locality and to one culture of each of the remaining kinds of plant material. In addition uninoculated controls and controls inoculated with a standard laboratory strain, strain *A*121111, [6]) were included, making in all 750 individual plant cultures.

The tubes were distributed entirely at random in 36 racks on two glasshouse benches, each rack consisting of a solid block of wood with 2 ranks of staggered holes 2″ deep and 2″ between centres to provide shade for roots. Each tube was numbered after randomization and temporary marks of strain and plant type were removed to avoid discrimination in scoring the plants in the course of the test. The plants were sown on 16th and 17th March and harvested between 20th and 28th July and were watered under sterile conditions four times during growth. The few tubes which became contaminated with fungus were discarded at harvest.

During the experiment observations were made of the response of the plants to nitrogen fixation. This was done by comparing the colour and size of each plant against five standard plants showing respectively: no response, a slight response, an intermediate response, a fairly good response and a normally effective response; the categories in increasing degrees of effectiveness were numbered 0, 1, 2, 3, 4. The standard plants were freshly selected at each time of scoring. At harvest nodules were counted and the dry weights were determined; in a second experiment nitrogen analyses were also made.

EXPERIMENTAL RESULTS

The experiment was designed principally to compare (1) the symbiosis of local strains of plant inoculated with their homologous bacteria (i.e. strains isolated from districts in which the parent plants were raised) with the symbiosis of the same plant material with heterologous bacteria (i.e. bacteria isolated from other districts) and to compare (2) the homologous symbiosis with the symbiosis of the local strains of bacteria and heterologous plant material. The Tables are set out to illustrate these comparisons and direct strain and plant differences.

Dry Weight

The response of individual plants was very variable. The histogram of dry weight for the whole of experiment 1 (Fig. 2) shows that the distribution of individual plant dry weight is skew. The great

majority of the plants show a more or less effective response, fewer are of intermediate effectiveness and only a small percentage show a wholly ineffective response. This distribution is exactly similar to that described for the response of a single kind of red clover with a pure line strain of bacteria [7]) in which it is shown that some of the ineffective plants formed a genetically distinct population. Here also there is a suggestion of a secondary mode in the ineffective class.

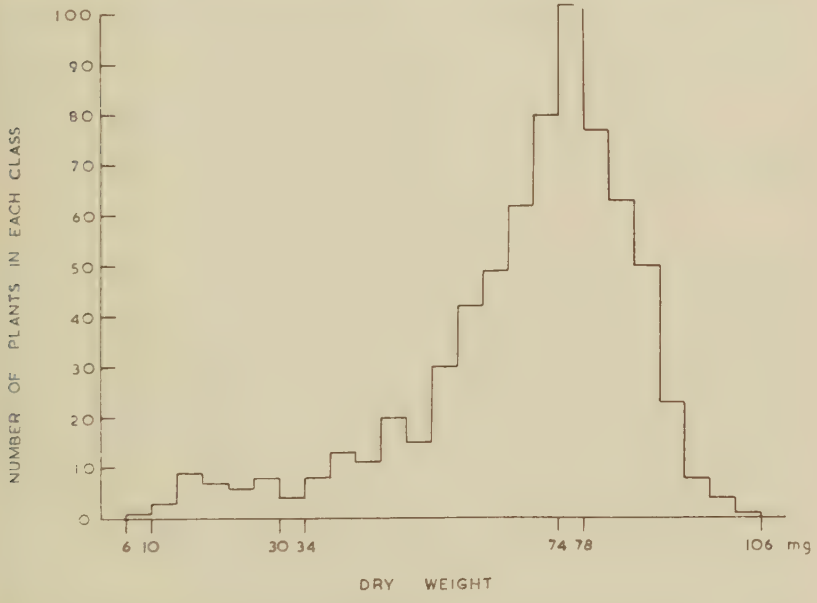

Fig. 2. Distribution of Dry Weight in Experiment I (all strains of Plant and Bacteria).

Analysis of the data shows that the variability is homogeneous in the experiment as a whole so that a general standard error may conveniently be used to test the significance of differences between means (S.E. per plant 14.62, 256 d.f.; 21.1% of mean).

General plant responses are set out in Table I which show that within the British material Cornish marl (*B*) and in the Swedish material Merkur (*P*) are most effective. It also appears that the strain *T* (Offer) and *U* (Kusträsk) are slightly less effective than *S* (Ultuna) and *P* (Merkur); no other differences are significant.

A comparison of the effectiveness of the different groups of isolates is made in Table II, and it is clear that there are no significant

TABLE I

The Mean Effectivity of each Local Strain of Clover in Symbiosis with all strains of Bacteria		
Local Clover Strain	Mean Dry Weight in mg	
British *A*	67.13	± 1.67
B	75.11	± 1.67
C	68.33	± 1.47
Swedish *P*	74.00	± 1.87
Q	68.52	± 1.71
R	66.15	± 1.77
S	70.11	± 1.76
T	64.59	± 1.89
U	69.55	± 1.77

Significant differences within British and Swedish material
$B > A$, and C
P and S, $> U$ and T
$P > R$

differences between any of the groups; and further analysis has shown that within each group there are no differences between siolates from the same or different plants. The detection of adaptation is made easier by the absence of bacterial strain differences. Adaptation can be assessed directly and it need only be borne in mind that the plant materials *B* and *P* are of rather greater effectiveness than the remainder.

TABLE II

The Mean Effectivity of each group of Local Isolates of Clover Nodule Bacteria in Symbiosis with all strains of Plant material		
Isolates of Bacteria	Mean Dry Weight in mg	
British *a*1–*a*7	70.09	± 1.45
*b*1–*b*8	67.83	± 1.46
*c*1–*c*12	67.99	± 1.17
Swedish *p*1–*p*4	69.69	± 2.09
*q*1–*q*4	69.67	± 2.09
*r*1–*r*4	69.80	± 2.09
*s*1–*s*4	71.16	± 2.05
*t*1–*t*4	71.21	± 2.11
*u*1–*u*2	69.54	± 2.09
*A*121111 Control	69.69	± 2.43
Uninoculated Control	18.70	

Comparison of Homologous and Heterologous Symbiosis

In Tables III, IV and V the mean dry weights are shown for the

principal groups of treatments; also shown are the mean differences between homologous and heterologous symbioses with their standard errors. In each case the heterologous means are subtracted from the homologous so that a positive difference indicates a larger mean dry weight in the latter.

TABLE III

Mean Effectivity Response (expressed as dry weight per plant) of Clover of British or Swedish Origin in Symbiosis with strains of Bacteria of similar or Foreign Nationality

Heterologous Bacteria	Homologous	Heterologous Plant	Mean Differences	
A—C, p—u	*A—C, a—c*	*P —U, a—c*		
69.85	70.10	66.89	0.25 ± 2.06	3.21 *) ± 1.55
P—U, a—c	*P —U, p u*	*A—C, p —u*		
66.89	70.28	69.85	3.39 *) ± 1.47	0.43 ± 2.01
67.74	70.20	67.74	2.46 *) ± 1.18	2.46 *) ± 1.18

*) $P = 0.05$
**) $P = 0.01$

(I) Between British and Swedish Material

The comparison between British and Swedish strains of plant and bacteria (Table III) shows that on the average a more effective symbiosis is formed between plants and bacteria of similar nationality. All the mean differences are positive and are statistically significant on the totals and in two of the four individual comparisons.

TABLE IV

The Response (Dry Weight per plant) of each British Local Strain of Red Clover in Symbiosis with strains of Nodule Bacteria isolated from its own locality (Homologous Symbiosis) and from other districts (Symbiosis with Heterologous Bacteria), and the Response of each Local Strain of Bacteria with other Local Strains of Plant Material (Symbiosis with Heterologous Plants)

Heterologous Bacteria	Homologous	Heterologous Plant	Mean Differences	
A, bc	*A, a*	*a, BC*		
72.30	66.93	69.30	—5.37 ± 4.03	—2.37 ± 3.97
B, ac	*B, b*	*b, AC*		
72.52	74.09	68.58	1.57 ± 4.05	5.51 ± 3.94
C, ab	*C, c*	*c, AB*		
69.37	67.59	74.80	—1.78 ± 3.39	—7.21 *) ± 3.49
71.15	69.34	71.15	—1.81 ± 2.19	—1.81 ± 2.19

(II) Within British Material

Within the British material (Table IV) there is no evidence of positive adaptation, indeed the overall difference between homologous and heterologous symbioses is negative; this is also the case if the two samples of Cornish marl coming from the same region are combined. The maximum positive difference occurs between the groups *B*, *b* and *b*, *AC*, and although this difference does not quite reach a level of significance it may be a reflection of the higher degree of effectiveness of *B* with all strains of bacteria, as noted in Table I. The symbiosis of the local strain of bacteria *c* with the homologous plant material is significantly less effective than with other strains of plant material.

TABLE V

Mean dry weights showing Effectivity response of Swedish Local Strains of Red Clover and of Local Isolates of Nodule Bacteria in symbiosis with Homologous and Heterologous strains of Bacteria and Plant respectively

Heterologous Bacteria	Homologous	Heterologous Plant	Mean Differences			
P, *q*—*u*	*P p*	*p*, *Q*—*U*				
68.12	77.23	63.65	9.11	± 4.99	13.58 **)	± 4.89
Q, *p*—*u* less *q*	*Q q*	*q*, *P*—*U* less *Q*				
66.90	72.01	65.61	5.11	± 4.53	6.40	± 4.47
R, *p*—*u* less *r*	*R r*	*r*, *P*—*U* less *R*				
70.51	66.31	74.44	—4.20	± 4.81	—8.13	± 4.81
S, *p*—*u* less *s*	*S s*	*s*, *P*—*U* less *S*				
69.55	75.73	67.23	6.18	± 4.68	8.50	± 4.68
T, *p*—*u* less *t*	*T t*	*t*, *P*—*U* less *T*				
67.06	72.92	69.90	5.86	± 4.88	3.02	± 4.75
U, *p*—*t*	*U u*	*u*, *P*—*T*				
67.14	72.30	68.96	5.16	± 4.83	3.34	± 4.83
68.15	72.62	68.15	4.47 *)	± 1.95	4.47 *)	± 1.95

(III) Within Swedish Material

Evidence for adaptation within the Swedish material is set out in Table V. In five of the six groups the homologous symbiosis is more effective than the heterologous. Ten of the twelve comparisons are positive and, of these, two approach a level of significance and one is highly significant. The latter involves the plant material *P* which

has been shown to be highly effective with all bacteria so that the difference between the symbiosis of the strain *p* with homologous and heterologous plants will be in part a simple plant effect. The difference, however, between the symbiosis of *P* plants with homologous and heterologous bacteria is also positive and approaches a level of significance. It may be noted that the high effectiveness of *P* will on the other hand, tend to obscure adaptation in half of the remaining comparisons.

Adaptation appears to be absent with the *R* (Sörby Okna) material due to the low level of the homologous symbiosis in which three very low values (17.6 mg, 29.0 mg and 31.4 mg) occurred; these plants were wholly ineffective. The main concern in this investigation is the response of the bulk of the population of more or less effectively responding plants. If it were possible to exclude the relatively small number of ineffectively responding plants the precision of the experiment would be improved and Sörby Okna would show local adaptation in one of the two comparisons. These ineffectives probably form a genetically distinct group which is elsewhere distributed more evenly among the treatments. Omitting the above three values the mean dry weight for the *Rr* becomes 73.86 mg. Further data on the position of *R* is given below in a confirmatory experiment.

Within the Swedish material as a whole there is thus local mutual adaptation of plant to bacteria. Both the relevant comparisons (without excluding the negative *Rr* material) indicate a positive adaptation which is significant at P 0.05 — 0.02. This average effect is small, amounting to 6.6% on a dry weight basis, but must clearly have been of significance in the adaptation of these local strains to their environment.

Nodule Number

The inverse correlation between the effectiveness of the response and the number of nodules on plants inoculated with strains of bacteria of markedly differing effectiveness is well established [9] and others). It is also known that individual plants differ in the number of nodules they are able to form; differences which are genetically determined and are to a large extent independent of bacterial strain [6]). In view of these relationships an analysis has been made of nodule number, the results are set out in Tables VI and VII.

TABLE VI

The Mean Number of Nodules formed on the Different kinds of Plant Material and by Bacterial Strains of Differing Origin	
1. Inoculated with all Strains of Bacteria	2. On all Strains of Plant
A 33.91 ± 2.50	a_1—a_7 38.68 ± 2.17
B 40.30 ± 2.50	b_1—b_8 35.10 ± 2.19
C 40.83 ± 2.20	c_1—c_{12} 43.91 ± 1.76
P 37.82 ± 2.80	p_1—p_4 34.98 ± 3.13
Q 45.08 ± 2.56	q_1—q_4 47.90 ± 3.13
R 38.12 ± 2.66	r_1—r_4 41.94 ± 3.13
S 42.41 ± 2.62	s_1—s_4 56.88 ± 3.07
T 43.97 ± 2.85	t_1—t_4 38.94 ± 3.16
U 54,85 ± 2.66	u_1—u_2 42.69 ± 3.13

Significant differences

$U > P, Q, R, S, T$	$s > p, q, r, t, u$
$Q > P, R$	$q > p, t$

TABLE VII

Mean Numbers of Nodules formed by Swedish Local Strains of Clover inoculated with Homologous and Heterologous Strains of Bacteria						
Heterologous Bacteria	Homologous	Heterologous Plant	Mean Differences			
P, q—u	*P p*	*p, Q—U*				
45.40	30.87	38.95	—14.53	± 7.48	— 8.08	± 7.33
Q, p—u less *q*	*Q q*	*q, P—U* less *Q*				
43.65	56.26	40.16	12.61	± 6.79	16.10*)	± 7.10
R, p—u less *r*	*R r*	*r, P—U* less *R*				
45.22	34.26	54.06	—10.96	± 7.20	—19.80**)	± 7.20
S, p—u less *s*	*S s*	*s, P—U* less *S*				
33.89	43.40	71.00	9.51	± 7.02	—27.60***)	± 7.02
T, p—u less *t*	*T t*	*t, P—U* less *T*				
59.22	43.33	34.95	—15.89 *)	± 7.30	8.38	± 7.12
U, p—t	*U u*	*u, P—T*				
55.25	48.00	45.00	— 7.25	± 7.23	3.00	± 7.23
46.96	43.03	46.96	— 3.93	± 2.92	—3.93	± 2.92

The number of nodules formed on a plant is an even more variable character than dry weight; the S.E. of a single plant is 21.89 nodules, 256 d.f.; 52.4% of mean.

Most of the plant and bacterial strains are indistinguishable (Table VI). Only the plant strain *U* and the bacterial strains s_1—s_4

differ significantly from all the remainder, and the plant *Q* and bacterial strain *q* are just significantly higher in nodule formation than *P* and *R* and *p* and *t* respectively. The other comparisons between treatments show no differences.

The data for the Swedish material in Table VII are arranged to show adaptation and are in marked contrast to the dry weight figures. With dry weight, the difference between homologous and heterologous symbioses is positive in the experiment as a whole; with nodule number it is negative. Of the twelve comparisons seven are negative and of these one is very highly significant, one highly significant, one significant and one approaching the level of significance. With the strain *Q*, however, adaptation appears to give rise to a larger number of nodules, a difference which is significant in the comparison *Qq* versus *q*, (*P* -*U* less *Q*). This however may be due to the tendency noted in Table VI for both *Q* and *q* to form a rather large number of nodules in all sets.

The data as a whole support the view that adaptation tends to a reduction in the number of nodules formed in the homologous symbiosis and is therefore in agreement with the finding that in general more effective symbioses occur with sparse nodulation.

Grading

The plants were graded on five occasions during the experiment; the results for the Swedish material are shown in graphical form in Fig. 3.

At each time of grading the differences between homologous and heterologous symbioses have been added together, a higher value for the homologous association being regarded positively. The date of sampling is used as abscissa. Most of these differences are positive and show a tendency to increase during the course of the growth of the plants. These results show that although the differences ascribed to adaptation are small they can be detected from a fairly early seedling stage (4 5 leaves) by a simple scoring system, provided a large enough sample of plants is available for examination.

Confirmatory Experiment

In 1949 a second experiment was set up using the following Swedish local plant strains: *P, R, T, U.*

As no differences appeared in the first experiment between parallel isolates of bacteria from each locality, one isolate only was used. A randomised block design was adopted, otherwise the experimental procedures were the same. Each of the ten blocks contained four replicates of the homologous treatments and one each of the remainder. The experiment was sown on April 4th and on 17th June and 6th July each plant was matched against standard plants. The experiment was harvested block by block from 10th–20th July, the plants being dried at 100°C for nitrogen determinations.

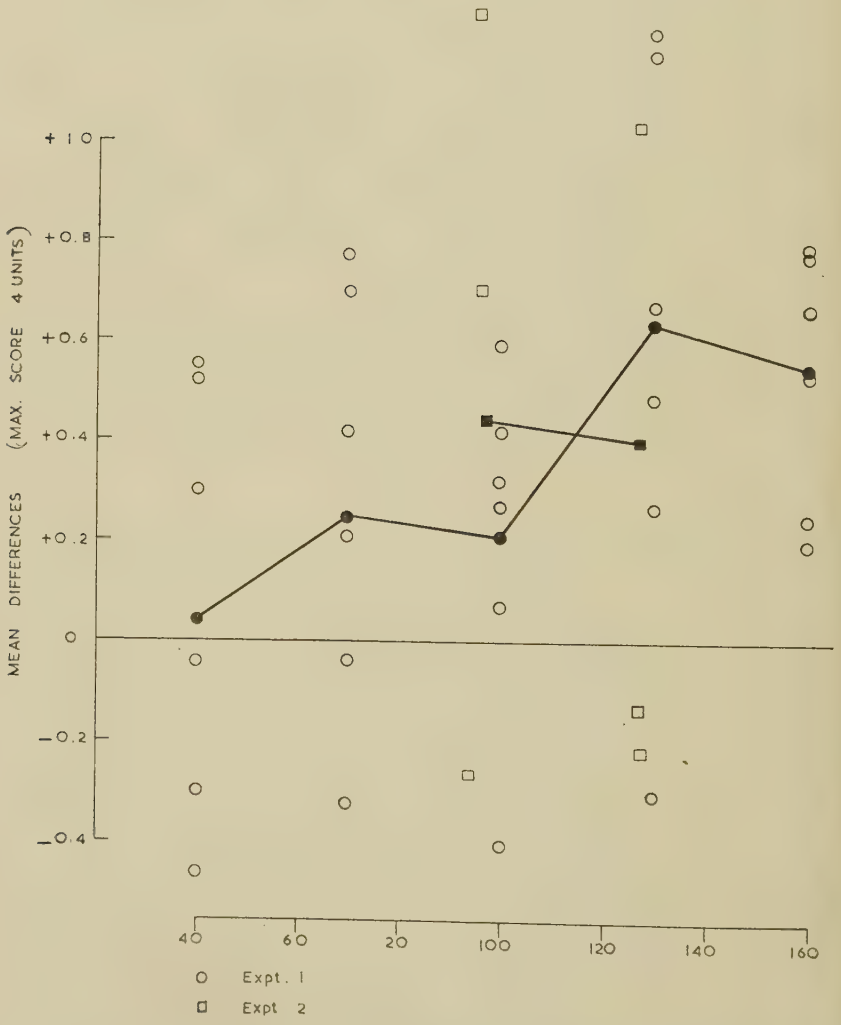

Fig. 3. Sum of Mean Differences between Homologous and Heterologous Symbioses for each group of Swedish strains of Clover and Bacteria.

Table VIII shows average strain and plant differences in dry weight, nitrogen content and number of nodules per plant. Variability in dry weight is rather less than over the whole of the first experiment (S.E. — 8.365 mg per plant). With the exception of *T* the order of increasing dry weight among the plant types is the same as in experiment 1, and the differences between them are small. Between bacterial strains, however, two interesting differences not observed in experiment 1 appear: *t* is very much less effective than the remainder and *u* is a little less so than *p* and *r*. The small difference between *u* and the remaining 48 isolates in experiment 1 is not apparent but it is clear that the effectiveness of *t* had decreased substantially during subculture. Under ordinary conditions subculture is generally without effect, but in this case, due to an oversight the cultures were at one stage allowed to nearly dry out and this may have been a contributory cause to the dissociation. Very unfavourable conditions of growth such as desiccation have been shown to favour the appearance of ineffective mutants in *Rhizo-*

TABLE VIII

Mean Effectivity Response (Dry Weight per Plant) Nitrogen Content and Number of Nodules formed on each strain of Plant and by each Bacterial Isolate

Local strain of clover or bacterial isolate	Dry Weight mg per plant	Nitrogen mg per plant	Nodule Number
Approximate S. E. mean	S. E. ± 1.29	S. E. ± 0.16	S. E. ± 57.95
P	58.06	1.94	48.30
R	56.04	1.77	45.47
T	63.05	—	58.76
U	56.12	1.65	63.90
p	60.59	1.76	31.33
r	63.59	1.74	48.00
t	50.54	—	76.73
u	58.77	1.48	60.38
Uninoculated control	19.10	0.31	—

Significant differences: (dry weight) $T > P$, R and U, p and $r > u > t$; (nitrogen), P and $R > U$, p and $r > u$; (nodule number) none.

bium [6]). One other strain (s_4) had become completely ineffective during the same period giving a dry weight of 19.09 mg and a nodule number of 130.4 per plant compared with 74.46 mg and 40.8 nodules respectively in experiment 1.

The number of nodules in this experiment is as variable as in

experiment 1 and reflects the differences noted between *t*, and to a lesser extent *u*, and *p* and *r* in dry weight; the least effective strain inducing the formation of the largest number of nodules. It may also be noted that the lowered effectiveness of *t* has resulted in a larger number of nodules on all types of plant material than was obtained in experiment 1.

The markedly reduced effectiveness of *t* makes the detection of adaptation more uncertain since it accentuates differences of the type *Rr* versus *R*, *ptu* at the expense of the *Tt* versus *t*, *PRU* comparison. The *T* and *t* material is therefore excluded from the main comparisons shown in Table IX and is entered separately below.

TABLE IX

Table of Mean Dry Weights and Mean Differences for Experiment 2

Heterologous Bacteria		Homologous	Heterologous plant		Mean Differences	
					$Pp-\frac{1}{2}(Pr-Pu)$ etc.	$Pp-\frac{1}{2}(pR-pU)$ etc.
Pr	*Pu*	*Pp*	*pR*	*pU*		
62.08	56.58	65.91	59.13	56.21	6.58 **) ± 2.89	8.24 **) ± 3.12
Rp	*Ru*	*Rr*	*rP*	*rU*		
59.13	52.08	64.80	62.08	61.68	9.20 **) ± 2.89	2.92 ± 2.89
Up	*Ur*	*Uu*	*uP*	*uR*		
56.21	61.68	58.52	56.58	52.08	—0.42 ± 2.74	4.19 ± 2.74
57.96		63.21	57.96		5.25	5.25
Data for T and t						
T, p r u		*Tt*	*T, PRU*			
66.81		51.79	49.95		—15.02	1.84

Because of the change in design a more complete analysis has been undertaken than in the first experiment; block differences are insignificant. Five of the six comparisons between homologous and heterologous symbioses (Table IX) show positive differences of which three are highly significant. Here, in contrast to experiment 1, the *R* material shows adaptation confirming the suggestion already made that the negative result first obtained was due to the fortuitous inclusion of three very low values in the homologous set. The small negative difference between *Uu* and *Urp* may be due to the slight reduction in the effectiveness of *u*, noted above; although here also the homologous set contains the lowest value in the experiment (29.0 mg).

Non-orthogonality, due to calculation of missing plots prevents

the ready application of a standard error to the overall differences; these however, are of the same magnitude as in experiment 1.

With *Tt* material the low effectiveness of *t* results in negative adaptation in the comparison *Tt* versus *T, pru*. The other comparison shows a positive difference in dry weight due wholly or in part to the greater effectiveness of the response of *T* independently of bacterial strain. As in experiment 1 adaptation may be detected by scoring plants for size and colour; the results have been inserted in Fig. 3 and lie close to the values obtained in the first experiment.

Nodule Number

Analysis of the results has been made on transformed data ($100 \times \log_{10} n + 1$) and are set out in Table X. As for dry weight no

TABLE X

Table of Mean Nodule Number and Mean Differences for Experiment 2
(Transformed to $100 \log_{10} n - 1$)

Heterologous Bacteria		Homologous	Heterologous plant		Mean Differences			
					$Pp - \frac{1}{2}(Pr - Pu)$ etc.		$Pp - \frac{1}{2}(pR - pU)$ etc.	
Pr	*Pu*	*Pp*	*pR*	*pU*				
160.1	154.9	143.4	128.1	139.0	−14.1	± 8.21	−10.0	± 8.86
Rp	*Ru*	*Rr*	*rP*	*rU*				
128.1	170.1	155.6	160.1	163.6	6.5	± 8.21	− 6.25	± 8.21
Up	*Ur*	*Uu*	*uP*	*uR*				
139.0	163.6	191.5	154.9	170.1	40.2**)	± 7.79	29.0**)	± 7.79

block differences are significant. Differences between homologous and heterologous symbioses are negative in half the comparisons only, compared with a more pronounced negative trend in the first test. This is because *U* and *u* both tend to produce a large number of nodules with all strains of bacteria and on all kinds of plant. The plant material *U* has shown this tendency in both experiments whereas *u* has clearly become more infective and less effective and appears to have subtained a mutative change similar to *t* but less marked.

Nitrogen Content

Micro-Kjeldahls were carried out *) on bulked samples and show

*) These estimations were undertaken through the kindness of Mr. R. G. Warren of the Chemistry Department.

no general trends in the amount of nitrogen fixed per plant in the homologous and heterologous sets (Table XI).

TABLE XI

Analysis of Nitrogen Content (mg per plant)				
Heterologous Bacteria	Homologous	Heterologous plant	Mean Differences (S. E. of Difference ± 0.1798)	
P, *r* and *u* 1.96	*Pp* 1.91	*p*, *R* and *U* 1.61	—0.05	0.30
R, *p* and *u* 1.80	*Rr* 1.74	*r*, *P* and *U* 1.74	—0.06	0
U, *r* and *p* 1.84	*Uu* 1.46	*u*, *R* and *P* 1.50	—0.38	—0.04
1.87	1.70	1.65	(S. E. of Difference ± 0.1615) —0.17	0.06

The results as a whole suggest that the adaptation which results in the growth of larger plants in homologous sets is not due to higher nitrogen fixation but to more efficient ultilisation of the nitrogen fixed or to stimulation of growth unconnected with nitrogen fixation.

DISCUSSION

These results with red clover are in agreement with the studies, noted above, on species of *Trifolium*, *Medicago* and *Melilotus* in showing that the effectiveness of the response in symbiosis depends upon the interaction of strain and host factors. Were this not so only direct bacterial and host effect need be considered, and in a few instances the effectiveness of symbiosis may be adequately described in such simple terms. Thus in experiment 2 the *U* plant material independent of strain and the *u* bacteria independent of plant type are of low effectiveness. In combination the influences of strain and host are supplementary and produce an even less effective symbiosis. In much of the rest of the material, however, plant and strain interact to give a symbiosis which is not related simply to individual plant and strain characteristics. The interactions in this material are, as might be expected, much less striking than those obtained in interspecific studies. These experiments have been designed to study particular interactions only, i.e. those related to the geographical origin of the material, and here two types of interaction have been demonstrated:

(1) Sporadic association between plant and bacteria leading to negative adaptation; the single example of this kind of interaction is given by the symbiosis of the English strain of bacteria *c* with *C* plants which shows a significantly less effective response than with plants of *A* or *B* type.

(2) A more general association, in the unique material available in Sweden, leading to positive adaptation between local strains of plant and bacteria; the local strain of plant making more growth in association with the local strain of bacteria than with other strains.

The data show that the adjustment is mutual, each organism becoming modified presumably by selection towards the raising of the efficiency of the symbiosis. Selection to this end may operate in the field through increased growth of the plant and by increasing the number of bacteria that may be released from larger and more effective nodules.

It is of interest that this relationship holds only with the Swedish material. In Britain where local strains are not so clearly defined, and where registration of local strains is not established, there is no evidence of positive adaptation; Cornish marl giving as effective a symbiosis with isolates from Montgomeryshire as with local bacteria.

In Sweden adaptation may be due to longer periods of isolation of the local strains of plant or to the much wider range of climatic conditions there. In this connection it should be noted that the local Swedish strains have been developed as far north as the Arctic circle and as far south as lat. 56°, and are spaced along climatic gradients of temperature, rainfall and growing period all of which follow a NNE—SSW direction.

The data have been re-examined to test whether the degree of adaptation is related to the distance separating the localities from which the plant and bacteria were derived. This has been done by assuming that the adaptation (W_{ij}) is proportional to the latitude difference (d_{ij}) between stations i and j.

i.e. that $$W_{ij} = A + B_i + H_i - Kd_{ij} + Z_{ij}$$

where A and K are constants, B_i and H_i are components contributed by bacterial strain and host factors and the residual term Z_{ij} represents error. The analysis of variance for each experiment in Table XII shows that the mean square due to K is not significant; i.e. the

data do not support the hypothesis that the adaptation is proportional to the latitude difference.

TABLE XII

Test of Relationship of Adaptation to Geographical Position, Analysis of Variance

Source	*DF*	Sum sqs.	Mean sq	Ratio
Experiment 1				
Bacteria	5	299.1351		
Plant	5	57.1555		
Due to *K*	1	121.4207	121.4207	1.68, $P > 20\%$
Error	24	1737.8677	72.4112	
Total	35	2215.5790		
Experiment 2				
Bacteria	4	7063.5328		
Plant	4	109.0887		
Due to *K*	1	30.1566	30.1566	2.82, $P > 10\%$
Error	15	106.6001	10.7067	
Total	24	7363.3782		

The positive intra-national adaptation is of interest in being chiefly due to the difference between Swedish and British plant material in symbiosis with British strains of bacteria, and to the British and Swedish strains of bacteria in association with British plant material. On British plants all strains of bacteria are equally effective and with Swedish bacteria all plants are of the same effectiveness. This may reflect the slightly higher effectiveness of Swedish isolates on all plant material (Table II).

The differences due to adaptation are small (about 5% dry wt. increase in each experiment) and are probably insignificant from the plant breeding point of view, and are unlikely to have any influence on seed inoculation unless augmented by other environmental factors. It is clear, however, from these results that where symbiosis is operating the adaptation of the cultivated plant is not only with respect to soil, climate and management but also with the associated microorganisms.

SUMMARY

A study has been made of the response of a number of local strains of British and Swedish red clover in symbiosis with nodule bacteria isolated from the localities from which the plant strains were obtained. All combinations of bacterial isolate and plant type were examined under bacteriologically controlled conditions of plant culture on an agar medium.

In the Swedish material each local strain of clover responded most effectively with bacteria isolated from its own locality. This adaptation was detected at about 8 weeks from planting by scoring the seedlings for size and colour, and at harvest was reflected in a mean difference of 5% on a dry weight basis. No differences were found in nitrogen content.

No adaptation was found within the British material possibly because of the more uniform climatic conditions. A small intra-national adaptation was significant.

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REFERENCES

1) Åkerberg, E. and Julén, G., Vart svenska rödklöver-material i belysning av utförda stamförsök. Kgl. Landtbruks. Akad. Tid. **85**, 541–593 (1946).
2) Bjälfve, G., Baljväxternas rotknölar hos olika sorter, baljväxternas kvävehalt samt deras kvävehushållning i akerjorden. Centralanst medd. 455 (1935).
3) Jensen, H. L., Nitrogen fixation in leguminous plants. I. General characters of root-nodule bacteria isolated from species of medicago and trifolium in Australia. Proc. Linnean Soc. N.S. Wales **67**, 98–108 (1942).
4) Kleczkowska, J., A study of phage-resistant mutants of Rhizobium trifolii. J. gen. Microbiol. **4**, 298–310 (1950).
5) Nutman, P. S., Variation within strains of clover nodule bacteria in the size of nodule produced and in the "effectivity" of the symbiosis. J. Bact. **51**,411–432 (1946).
6) Nutman, P. S., Genetical factors concerned in the symbiosis of clover and nodule bacteria. Nature **157**, 463 (1946).
7) Nutman, P. S., The interaction of hereditarily determined strain and host factors in symbiosis. Fourth Int. Congr. Microbiol. Copenhagen 474 (1947).
8) Nutman, P. S., The influence of strain and host factors on the efficiency of nitrogen fixation in clover. Brit. Commonwealth Sci. Offical Confer. Australia (1949).
9) Stevens, J. W., A study of various strains of Bacillus radicicola from nodules of alfalfa and sweet clover. Soil Sci. **20**, 45–66 (1925).
10) Thornton, H. G., The influence of the host plant in inducing parasitism in lucerne and clover nodules. Proc. Roy. Soc. B. **106**, 110—122 (1930).
11) Vincent, J. M., Host specificity amongst root-nodule bacteria isolated from several clover species. J. Australian Inst. Agr. Sci. **11**, 121–127 (1945).
12) Wilson, P. W., Burton, J. C. and Bond, V. S., Effect of species of host plant on nitrogen fixation in Melilotus. J. Agr. Research **55**, 619–629 (1937).

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ROOT-KNOT NEMATODES

III. EFFECTS OF *MELOIDOGYNE INCOGNITA* AND *M. JAVANICA* ON SOME PEACH ROOTSTOCKS

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INTRODUCTION

For many years the problem of root-knot nematode damage has been one of the serious ones to peach growers and nurserymen in the more southern districts of the United States. Although nematodes rarely cause death of the trees directly, they weaken the trees to such an extent that they are easily destroyed by other factors. Winter injury, drought, poor soil drainage, or other factors cause serious damage or death to trees whose roots are infected with nematodes. Various nematode control methods have been attempted, principally soil fumigation, use of relatively resistant understocks, and use of resistant cover crops. Fumigation methods have lacked permanence. The most resistant understocks now used have been found susceptible to certain nematodes, and the use of resistant cover crops often has not been practical from the standpoint of orchard soil management.

This is a report of a study of the relative susceptibility of six peach understocks to two of the principal species of peach root-knot nematodes, *Meloidogyne incognita* (Kofoid and White) Chitwood and *M. javanica* (Treub) Chitwood, and of effects of nematode infections on leaf composition. The characteristics and identification of these two species have been given in detail elsewhere [4]), but they are described briefly here, together with several others often associated with peach trees.

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Species of Meloidogyne Associated With Peach Trees

Meloidogyne incognita (Kofoid and White) Chitwood. This species is generally distributed throughout the southern United States and Mexico. It is apparently native as far north as North Carolina and overwinters as far north as New Jersey. Regardless of the source of this nematode, all populations reproduced moderately on Lovell variety peach and injured the seedlings of this variety when rootstocks were inoculated with large numbers of larvae.

Meloidogyne incognita var. *acrita* Chitwood. This organism was first described [4]) as a morphologic differentiation from *M. incognita*, but no differences in host range or pathology had been obtained. It was originally taken from Georgia cotton roots but was found to attack Lovell peach seedlings in a manner similar to *M. incognita*. Since the original publication, physiologic as well as pathologic differences have been obtained by J. H. Machmer, A. L. Taylor and A. C. Tarjan, of the Division of Nematology. It would seem reasonable to elevate this organism to subspecific rank, provided it is shown to be stable. It is certainly an organism to be reckoned with in varietal susceptibility testing.

Meloidogyne javanica (Treub) Chitwood. This nematode is irregularly distributed. It was originally described from sugarcane in Java. In this country it has been found attacking Yunnan and Shalil peach rootstocks in Florida, Georgia, and North Carolina. It was also collected from root-knot nematode test plots in California and Texas. Carnations from a Long Island greenhouse were found infected by this species. Regardless of source this species was found to reproduce moderately on the roots of Yunnan variety peach seedlings. At the present time it is uncertain whether this nematode represents a foreign introduction distributed on nursery stock or a recurring mutant of *M. incognita*. The high percentage of abnormal males and the occurrence of rare, laterally asymmetric females in this species indicate an unbalance which might be chromosomal.

Meloidogyne arenaria (Neal) Chitwood. This nematode is, as yet, known only from Florida and Georgia. Neal [20]) stated, "In a field near my place, heavily fertilized with bone and potash compound, the roots of the peanut became masses of knotty roots, the worst cases of the disease I ever saw, and peach trees growing in that field are ruined". Our attempts to establish this nematode on Lovell seedlings failed, although the roots were destroyed and many plants died.

A new subspecies of *Meloidogyne arenaria* was obtained from "*Boehmeria utilis*" (syn. of *B. nivea* (L.) Gaud.) from Florida and illustrated by Chitwood [4]) as *M. arenaria* from *Boehmeria* *). Recently it has been found to

*) Diagnosis of *Meloidogyne arenaria* subspecies *thamesi* new subspecies, by B. G. Chitwood is as follows. This nematode differs from the type *M. arenaria* morphologically in that the perineal pattern of the female shows a vertical series of transverse markings; that the knobs of the male stylet are asymmetric; that the head of the male tends to lie in a semi-oblique position; that the dorsal gland crifice is only 33μ behind

differ physiologically from *M. arenaria* in that it has not been possible to propagate successive generations on peanuts. Some 40 mass inoculations of peanuts resulted in the formation of 22 total egg masses. These were propagated on tomatoes and the resultant progeny was used in an additional attempt to inoculate peanuts. No survivors were obtained on peanuts though tomatoes, under the same conditions, were heavily infected. Thus, it appears that *M. arenaria* subspecies *thamesi* is not a selection of *M. arenaria*.

Meloidogyne hapla Chitwood*). This nematode has been found from Maine to North Carolina and as far west as North Dakota and New Mexico. It is present in nearly all commercial potato plantings within this territory. However, it is also found on a wide variety of hosts, including forsythia, strawberry, and peanut. Like *M. arenaria*, *M. hapla* is very destructive to Lovell peach roots but little, if any, reproduction occurs thereon. At very high rates of soil infestation by the nematode, significant growth differences in the plants were obtained as a result of the initial invasion. Eventually, however, in the absence of added inoculum, the plants outgrew the nematode injury.

In general, a surplus population of any species of root-knot nematode may damage or even kill a peach seedling even though the nematodes in particular may not reproduce thereon. This is an extremely important point in the interpretation of data in varietal tests or field experiments. It also bears on replanting problems and cover crops in peach orchards since root-knot nematodes propagating on other hosts may be, and often are, the major cause of poor peach growth.

Other Associates in Decline of Peach Trees

In addition to various root-knot nematodes of the genus *Meloidogyne* the senior author has found cases in which *Criconemoides simile* (Cobb) Chitwood, *Xiphinema americanum* Cobb, and *Belonolaimus gracilis* Steiner were associated with decline of peach trees. Each of these nematodes was the predominant nematode in the vicinity of the roots of certain declining trees in the sandy coastal plains area.

Criconemoides simile was screened out of soils from the Eastern Shore of Maryland [5]) and Lovell variety peach seedlings were inoculated with it. These seedlings showed chlorosis and a significant reduction in growth, with

the stylet base in both males and females; and that the larval tail is blunt, tending to terminate in bifid or trifid lobes. The rectum of the larva tends to be dilated, a characteristic, however, shared by another species, *M. incognita*. It is named after W. H. T h a m e s, assistant entomologist, Everglades Experiment Station, Belle Glade, Florida.

*) Footnote by B. G. C h i t w o o d. As mentioned in an earlier publication [4]), it is possible that *M. hapla* is a synonym of *Anguillula marioni* Cornu 1879. If so the name becomes *Meloidogyne marioni* (Cornu 1879) new combination. It was originally described from *Onobrychis sativa* Lam. (*O. viciaefolia* Scop.) from Chateauneuf-sur-Loire, France. It is impossible to recognize it specifically from the description.

leaf dropping from the base upwards. The nematode is apparently endemic in the sandy coastal plains from New Jersey to Louisiana and may have had pine trees as the original host. We have not succeeded in propagating it on the herbaceous plants tested. It is ectoparasitic and rather sedentary.

Xiphinema americanum was found to be the predominant nematode associated with declining peach trees in certain instances in Maryland. No experimental evidence of its pathogenicity is available. However, it is widespread in the eastern United States. It reproduces very slowly and is external and migratory.

Belonolaimus gracilis was found associated with decline in peach orchards near Holland, Virginia. It was also found associated with growth difficulties of peanuts in the same locality. Steiner [25]) originally described it as an associate of slash pine roots, *Pinus caribaea* Morelet, at Ocala, Florida. It was also found associated with the roots of corn, celery, strawberry, and other plants between Virginia and Florida. It is ectoparasitic and migratory; and since it often feeds on roots and moves to another location, it may do considerable damage to the roots.

Panagrolaimus spp. These organisms have been found inside decaying peach roots and coiled within apparently living cells where no decay had set in. Apparently members of the genus *Panagrolaimus* may feed on the products of decay. Whether they initiate decay or decomposition is an interesting question. It would certainly be remarkable for a saprophagous organism to enter plant roots and there set up a decay condition and feed on the products of its activities.

Miscellaneous fungi. There are often numerous fungi present in peach roots. In old root galls it is often difficult to decide whether fungi are parasitizing root-knot nematodes, mucoid egg masses of root-knot nematodes, root tissues, or all three.

REVIEW OF LITERATURE

Investigations on the resistance of peach rootstocks to root-knot nematodes were initiated in 1925. Tufts [28]), Tufts and Day [29]), Anonymous [1]), Hutchins [16]), Day and Tufts [12]) [13]), Christie and Havis [9]), and Havis *et al* [15]) reported on this extended study involving breeding, selection and testing of understocks. The earlier work was summarized by Tyler in 1941 [30]). Briefly, the findings with these rootstocks were as follows: Almonds (*Prunus amygdalus* Batsch) were variable in resistance and Japanese apricots (*Prunus mumem* (Sieb.) Sieb and Zucc.) were completely resistant but resulted in dwarfed peach trees. Hortulanum plum (*Prunus hortulana* Bailey) was highly resistant but gave dwarfed peach trees. *Prunus davidiana* (Carr.) Franch (source Nanking, China) was susceptible and dwarfed the peach trees, which

were susceptible to winter injury. "Natural" peach stocks from the southeastern United States mountain regions were quite vigorous but heavily infected in severely infested soil. Shalil variety peach introduced from India to Chico, California, was found to be vigorous and "completely resistant" at Fort Valley, Georgia, and relatively resistant in California. Day and Tufts[12] reported finding Shalil, Yunnan, and Bokhara varieties relatively resistant but with infected roots in some orchards. Because of the differences in root symptoms according to orchard, Day and Tufts hypothesized physiologically different races of root-knot nematodes. No apparent injury to top growth was encountered as a result of nematode infection of these rootstocks.

McBeth and Taylor[18] showed that Yunnan and Shalil varieties were more resistant to root-knot nematodes than the common rootstock. Their figure 2 shows better growth of Yunnan where nonsusceptible cover crops were used but one cannot be certain from their results whether the nematodes injuring the trees were propagated on peach roots or on the cover crop. J. R. Christie (unpublished observations) found that at best peach rootstocks were poor hosts for root-knot nematodes. Our own observations have been that root-knot nematodes propagate more slowly on susceptible peach root-stock than on most other susceptible host plants.

Christie and Havis[9] showed that root-knot nematodes from different sources behave differently on peach varieties. Machmer[17] showed that nematode-reducing rotations prior to planting and site fumigation increased the yield of peach trees. Chitwood[4] studied the root-knot nematodes of peach and other hosts and, upon finding morphological differences, placed them in several species and varieties.

In addition to their direct effects on the growth of trees, nematodes evidently may affect plant growth through their influence on chemical composition of plants. Information on relations of nematode infestations and internal nutrition of plants seems limited. The importance of nutrient balance in the interpretation of leaf composition as related to plant growth was emphasized by Shear, Crane, and Myers[23] and others. Goodall and Gregory[14] also pointed out that conclusions regarding growth based on the leaf composition of a single element are generally unsatis-

factory. Cooper[10]) and Thatcher[27]) suggested element groupings for studying nutrient interrelations.

Evidently little research has been done on the relative susceptibility of different peach rootstock varieties to the different species and varieties of nematodes. Even more lacking is specific information on the relation of nematode infestations and chemical composition of trees.

COMPLEXITY OF THE PROBLEMS

From the above discussion it is obvious that root problems in peach orchards are really multitudinous. Lack of exactness in use of terms, such as "hardiness", "vigor", and "winter injury" add to the problems. This indefiniteness of terms is somewhat parallel to the use of the term "physiological race" in nematology. Historically, we find that time after time physiological races have been hypothesized until someone learns to recognize the organisms morphologically. In the genus *Meloidogyne* (the root-knot nematodes) we apparently have limitless morphological diversity with a few major types. Some of these types appear to breed true, while others do not. Host specificity is incomplete[7]). Cases of absolute resistance in the sense of no reproduction of a given nematode on a given variety of plant are rare. Usually a small number of females of a given "race", "variety", "subspecies", or "species" produce eggs on a plant which might be characterized as a "non-host". In a few hundred experimental cases these few propagating females died out after the first or second generation. Do we have here the mechanism of natural selection? A billion, billion chances of reproduction may occur in the field every year. It behooves us to exert extreme caution in "recommending" "resistant" varieties.

In addition to the various root-knot nematodes, *Criconemoides simile* and *Belonolaimus gracilis* will eventually have to be given some consideration. The status of the other nematodes, fungi, etc., found associated with diseased peach roots will also require attention; otherwise we may find ourselves "recommending" rootstocks which resist one or more organisms but are even more susceptible to other diseases or troubles than is the usual planting stock.

The diagnosis of disease in the field and attribution of symptoms to specific organisms are also extremely difficult. Mixed field infestations of one or more root-knot nematodes and of other genera and

species of plant pathogenic nematodes, besides those of other organisms in soil, have to be segregated and the organisms examined individually and in combination before diagnoses of diseases can be considered final.

With all of this preliminary discussion, we feel the reader may be better prepared to consider our experimental data and suggestions, taking them with the necessary grain of salt.

EXPERIMENTAL RESULTS

Seedlings of five peach varieties and a hybrid were investigated as to their response to two species of root-knot nematodes. For the purposes of this paper the hybrid will be treated as a variety. The six are Lovell, Yunnan, Red Shadow, Davidson, S 37, and P. I. 146135 (Chico No. 16) × *Prunus davidiana* (C-26712). The origin of these stocks has been previously discussed by Havis et al.[15]). The root-knot nematodes used were *Meloidogyne javanica* and *M. incognita* [isolations 11C and 11D of Christie and Havis[9])]. The nematodes had been propagated in roots of tomato plants grown in the greenhouse. The roots of the plants were rinsed, chopped fine, and weighed; aliquot parts were examined to determine the number of egg masses per weighed unit of root inoculum. Weighed aliquots of soil with finely divided roots from the same plants were placed in water, decanted, and washed through a 200-mesh screen and the egg masses in the screened residues were also counted. Then each pot of recently steam-sterilized sandy loam was planted to a seedling only 5 to 8 cm high which had been grown from seed germinated in sand. The soil in each pot was inoculated at planting with known quantities of either *M. javanica* or *M. incognita*; in one of the experiments other pots of soil were left sterile as controls. Inoculum was expressed in terms of egg masses. At the end of the tests leaf samples were analyzed spectrochemically for B, P, Mg, Mn, Cu, Fe, Al, Ca, and Na by complete burning in a D.C. arc, using comparison standard technique [11]). K was determined with a flame photometer [19]). The tests here reported may well be presented as two experiments, (A) involving three varieties of peach seedlings inoculated with various quantities of *M. javanica* only and (B) inoculation of seedlings of six peach varieties with various quantities of both *M. incognita* and *M. javanica*.

Experiment A. – Inoculation of three varieties of peach seedlings with M. javanica at three dosage rates

Peach seedlings (one week old) were planted in 5-inch pots of inoculated sterilized soil in the greenhouse on March 8, 1949. Readings of plant growth and nematode production were taken on June 15, 1949 (table I).

In this experiment the peach variety S 37 did not show significant injury with any of the different amounts of inoculum tested. There appeared to be a minor (insignificant) increase in top weight with the moderate amount of inoculum (100 egg masses per plant) and a minor decrease in top weight with the larger amount of inoculum (370 egg masses per plant). The root weight was significantly increased with both medium and large quantities of inoculum. With the increase of inoculum from medium to large, the trend in root weight was downward. Although females were found in the roots, the rate of reproduction of this nematode on this variety of plant was extremely low as indicated by RX (rate of reproduction) of egg masses (table I).

The variety Red Shadow showed a clear decrease in both top weight and root weight with increase in quantity of inoculum (table I). This decrease was highly significant with the maximum quantity of inoculum. The rate of reproduction of the nematodes as indicated by both numbers of females and numbers of egg masses was usually much more than that on seedlings of the variety S 37. There was a decided decrease in the rate of reproduction with increase in quantity of inoculum. This effect, first noted by Carrol [2]) and Carrol and McMahon [3]) and later explained by Chitwood and Feldmesser [6]) for the golden nematode of potatoes, appears to be associated with a saturation level of the population of the nematode in relation to quantity of root. When significant reduction in root weight occurs, we invariably find that the nematode population falls. In other words, the nematodes do not reproduce themselves. On the basis of these facts and the results of this test it is doubtful that *M. javanica* propagates sufficiently in roots of the variety Red Shadow to cause damage (table I).

The variety Yunnan exhibited a highly significant incresae in top weight as a result of the moderate amount of inoculum and a significant decline as a result of the maximum quantity of inoculum. There was no significant difference in root weight with the moderate amount of inoculum, but a highly significant reduction with the

TABLE I

Effect of inoculation of seedlings of 3 varieties of peach with *Meloidogyne javanica*. (Planted Mar. 7, 1949; examined June 15, 1949; five seedlings of each variety inoculated with each quantity of inoculum)

Variety	Inoculum (egg masses)	Top weight	Root weight	Nematodes resulting: Females per gram of roots	Females per plant	Egg masses per gram of roots	Egg masses per plant	RX of females	RX of egg masses
	Number	Grams	Grams	Number	Number	Number	Number		
S 37	10	32 ± 4.2	15 ± 2.5	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	100	39 ± 2.8	30 ± 2.6 [2]	7 ± 3.3	201 ± 95	1.0 ± 0.6	28 ± 17.4	2.0 ± 0.9	0.28 ± 0.17
	370	25 ± 4.5	25 ± 3.3 [1]	20 ± 7.8	442 ± 123	1.0 ± 0.6	20 ± 12.8	1.14 ± 1.09	0.05 ± 0.03
Red Shadow	10	38 ± 2.4	23 ± 2.0	3.5 ± 1.3	71 ± 29	3.0 ± 1.4	64 ± 29	7.1 ± 2.9	6.4 ± 3.2
	100	32 ± 3.9	21 ± 2.0	30 ± 2.6	605 ± 259	4.5 ± 1.4	97 ± 41	6.0 ± 3.6	0.97 ± 0.4
	370	19 ± 2.2 [2] [3]	12 ± 1.3 [2] [4]	20 ± 4.2	241 ± 71	4.0 ± 2.0	57 ± 24	0.65 ± 0.19	0.12 ± 0.026
Yunnan	10	38 ± 4.0	13.4 ± 1.2	28 ± 14	295 ± 113	19 ± 11	179 ± 91	29.5 ± 11	17.9 ± 9.1
	100	56 ± 2.4 [2]	14.8 ± 0.2	44 ± 15	673 ± 252	13 ± 7	204 ± 109	6.7 ± 2.5	2.04 ± 1.09
	370	28 ± 8.6 [3]	6.4 ± 2.2 [2] [4]	91 ± 26	508 ± 226	22 ± 11	102 ± 28	1.3 ± 0.14	028 ± 0.08

[1]) Significantly differs from 10 egg masses of inoculum at .05 level.
[2]) ,, ,, ,, 10 ,, ,, ,, ,, ,, .01 ,,
[3]) ,, ,, ,, 100 ,, ,, ,, ,, ,, .05 ,,
[4]) ,, ,, ,, 100 ,, ,, ,, ,, ,, .01 ,,.
RX = Rate of reproduction of nematodes.

maximum quantity of inoculum. The rate of reproduction in terms of both females and egg masses was much greater in this variety than in either of the others. Decline in rate of reproduction was correlated with increase in amount of inoculum. Ten egg masses produced 179 egg masses and 100 egg masses produced 204 egg masses. Whether the 179 to 204 egg masses so produced would be sufficient to cause growth reduction of the new roots exposed is questionable. It would appear that here there is a very delicate balance between rate of plant growth and rate of nematode reproduction. In the field, certainly a very little reproduction of this nematode on a cover crop would be sufficient to cause reduction in peach root growth. The interdependence of quantities of root, top, and nematodes at a given time has previously been discussed [4] [7] [8].

Random selections of leaves of plants of the varieties Red Shadow and Yunnan were analyzed spectrochemically for ten elements. The information obtained from these analyses is summarized in table II. There was a notable increase in the total parts per million (sum of all elements determined) associated with increase in quantity of inoculum. This increase was primarily due to increase of K, or Ca, or both, but there was a comparable increase in B. Distinct decreases in Mg and Fe were associated with increase in quantity of inoculum. Decrease in absorptive surface as indicated by the weight of roots was associated with increase of inoculum. It seems possible that the lack of decrease in K content may be a result of the ease of entrance of this material with an adequate water supply in spite of poor root formation. The plants exposed to the maximum inoculum showed

TABLE II

Leaf composition of two varieties of peach seedlings infected with *M. javanica*, planted March 8, 1949 and sampled June 15, 1949

Variety	Inoculum (egg masses)	Root weight	Parts per million as determined spectrographically										
	Number	Grams	Cu	Fe	Al	Mn	B	P	Na	Mg	Ca	K	T.I.I.N.
Red Shadow	10	23 ± 2.0	6.9	414	332	279	58.2	2,460	196	3,087	5,908	16,625	29,366
	100	21 ± 2.0	5.4	232	184	291	67.2	2,204	188	2,650	6,750	19,570	32,143
	370	12 ± 1.3	4.5	165	247	259	79.0	2,437	174	2,287	7,025	24,500	37,178
Yunnan	10	13.4 ± 1.2	8.1	231	162	275	21.5	1,708	166	2,117	4,125	18,250	27,064
	100	14.8 ± 0.7	39	190	172	194	22.5	1,934	220	1,975	8,600	16,625	29,972
	370	6.4 ± 2.2	6.2	107	130	192	26.0	1,900	162	1,775	6,567	23,000	33,865

[1]) Total intensity of internal nutrition (sum of all elements determined).

distinct chlorosis with leaf dropping from the bottom upward. This chlorosis appears to have been due to deficiency of Mg or Fe or both of these elements. Both elements have been demonstrated in the nematode body. Furthermore, it is a well-known fact that iron is an important factor in animal metabolism as a part of the cytochrome enzymes. The chlorosis of the plants may be due to injury to roots, which may cause difficulty in the absorption of these elements, or to differential absorption of Fe or Mg or both by the nematode. Deficiency in Fe is supposed to prevent use of Mg in chlorophyll formation. The often noted relative increase in K may also be partly due to the plant's substitution of K for Mg.

Experiment B. Inoculation of seedlings of six peach varieties with various quantities of Meloidogyne incognita or M. javanica

Week-old seedlings of six peach varieties were planted in the greenhouse on February 28, 1949, in 5-inch pots of sterilized soil, inoculated with varying numbers of egg masses prior to planting. Early readings of plant growth (March 28, 1949) were made on the basis of heights of seedlings. At that time stunting (table III, height) and chlorosis were usually obvious on plants inoculated with one or both species of nematodes. There was an immediate effect of the nematode inoculum on the host, which differed considerably from the effect produced by subsequent generations of nematodes. The injury, which was often very apparent, may have merely been due to extreme root destruction. Hence, final records were delayed until September 15, 1949. At that time root-gall indexes and the weights of tops and roots of the plants were recorded. The standard procedure for the former consists of visually grading the root system for amount of galling: Grade 0, no trace of galls; 1, trace of galls (1–2 galls); 2, few scattered galls; 3, moderate galling, galls general through root system; 4, moderately heavy galling; 5, galling extreme. Weighed root aliquots were examined and the results were reported by Davis et al.[15]. The tops were used to obtain leaf samples for spectrographic analyses and consideration of the leaf composition at time of harvest.

It is particularly striking that in September the variety Lovell showed a significant increase in top growth as a result of the smaller quantity of *M. javanica*. Furthermore, the variety Yunnan exhibited highly significant increases in top growth as a result of both the

TABLE III

Effect of inoculation seedlings of 6 varieties of peach with *Meloidogyne incognita* and *M. javanica* (Planted February 28, 1949; examined March 28 and September 15, 1949)

Peach variety	Seedlings	Nematode	Inoculum (egg masses)	Early record of height (Mar. 28)	Late record (Sept. 15)		
					Weight		Root Gal index [1]
					Tops	Roots	
	Number		Number	Cm	G	G	
Lovell	5	None (control)	0	13.7 ± 1.1	56 ± 6	55 ± 7	0.2
	6	*M. incognita*	123	6.2 ± 0.7 [3]	55 ± 3	54 ± 6	3.0
	5	,,	442	5.0 ± 0.6 [3]	45 ± 3	31 ± 7	3.6
	6	*M. javanica*	160	12.1 ± 1.3	86 ± 8 [2]	76 ± 10	2.0
	4	,,	515	6.9 ± 1.1 [3]	49 ± 12	48 ± 6	2.6
Yunnan	6	None	0	15.2 ± 2.2	70 ± 1	70 ± 4	0.0
	6	*M. incognita*	123	15.0 ± 1,5	102 ± 8 [3]	85 ± 9	0.8
	4	,,	442	11.3 ± 1.1 [2]	94 ± 4 [3]	79 ± 12	0.8
	6	*M. javanica*	160	14.3 ± 1.7	77 ± 7	77 ± 8	4.0
	4	,,	515	6.1 ± 0.8 [3]	70 ± 4	34 ± 3 [3]	2.2
S 37	6	None	0	20.2 ± 1.0	65 ± 10	59 ± 7	0.0
	6	*M. incognita*	123	22.4 ± 1.7	77 ± 5	72 ± 13	1.5
	3	,,	246	15.6 ± 1.0 [3]	81 ± 7	62 ± 2	1.1
	4	,,	442	23.6 ± 1.7	75 ± 9	59 ± 9	1.1
	5	*M. javanica*	160	11.2 ± 1.3 [3]	77 ± 11	78 ± 7	1.2
	6	,,	320	20.8 ± 1.0	61 ± 12	135 ± 5 [2]	1.1
	3	,,	515	20.7 ± 1.5	82 ± 12	40 ± 8	1.8
P.I. 146135 × *Prunus davidiana*	6	None	0	16.6 ± 2.4	68 ± 3	63 ± 6	0.0
	4	*M. incognita*	123	8.7 ± 2.1 [2]	59 ± 8	47 ± 4	0.7
	5	,,	442	10.7 ± 2.7	60 ± 5	43 ± 8	0.8
	4	*M. javanica*	160	9.2 ± 2.8	52 ± 9	47 ± 12	2.4
	4	,,	515	7.1 + 1.5 [3]	40 ± 14	38 ± 5	1.8
Red Shadow	6	None	0	16.0 ± 3.3	53 ± 8	63 ± 12	0.2
	2	*M. incognita*	246	17.5 ± 2.8	55 ± 10	97 ± 27	3.6
	5	,,	442	15.7 ± 0.7	50 ± 10	90 ± 10	3.7
	2	*M. javanica*	320	9.2 ± 0.6	71 ± 6	48 ± 8	2.8
	3	,,	515	8.7 ± 0.7	80 ± 6	46 ± 6	1.9
Davidson	6	None	0		76 ± 5	91 ± 6	0.0
	5	*M. incognita*	123	19.5 ± 1.5	51 ± 4	61 ± 5	3.5
	2	,,	246	23.2 ± 1.0	88 ± 12	70 ± 4	3.7
	5	*M. javanica*	160	18.7 ± 1.2 [2]	82 ± 4	60 ± 3	3.0
	2	,,	320	15.0 ± 1.4 [3]	85 ± 5	76 ± 19	2.1

[1] Relative amount of galling from 0 (none) to 5 (extreme).
[2] Significantly differs from control at .05 level.
[3] ,, ,, ,, ,, .01 ,,

smaller and the larger amount of inoculum of *M. incognita* (table III).

Root weights present another picture. The variety Yunnan showed a very highly significant decrease in root weight with the

large quantity of inoculum of *M. javanica* (table III). The variety S 37 exhibited a significant increase in root weight with the moderate amount of *M. javanica*. None of the other differences were significant in September. The general trend was toward lowest root weight in the pots having the largest quantity of inoculum, but the varieties of peaches differed in their response to the two species of nematodes. The cross, P.I. 146135 × *Prunus davidiana*, showed no evidence of root galling with any quantity of either species of nematode. From a horticultural standpoint this may be important.

The root-gall index indicated that the variety Lovell was susceptible to both species of root-knot nematodes used. The varieties Red Shadow and Davidson showed no evidence of resistance but considerable tolerance (able to grow well in spite of nematodes). The variety Yunnan exhibited a high degree of resistance to *M. incognita*, but none to *M. javanica*. Of the six peach varieties tested S 37 exhibited the most combined resistance to the two nematode species in the tests (table III, root-gall index). According to the previous report by Havis *et al.*[15]) both species of nematode reproduced well on Lovell. On the variety Red Shadow *M. incognita* reproduced moderately, but *M. javanica* did not reproduce. On the variety Davidson *M. incognita* reproduced moderately, but there was only a trace of reproduction by *M. javanica*. On P.I. 146135 × *P. davidiana* *M. incognita* did not reproduce, but *M. javanica* reproduced in small numbers. On S 37 there was a trace of reproduction by *M. incognita*, but *M. javanica* did not reproduce. Subsequently it has been found that *M. javanica* also reproduces to a minor extent on S 37. On the variety Yunnan *M. incognita* did not reproduce, but *M. javanica* reproduced moderately. Conclusions based on root-gall index (table III) would differ considerably from those arrived at by microscopic examination. It is quite obvious that root galling is not a basis for determining lack of resistance.

The information from the spectrographic analysis of the leaves of the varieties Lovell, Yunnan, and S 37 is summarized in table IV. The most obvious alteration in leaf composition was an increase in the K concentration associated with inoculation with any of the three varieties of peach seedling with either species of nematode. There was also a distinct decline in concentration of Mg in the susceptible variety Lovell associated with inoculation by both nematode species. Likewise the variety Yunnan, which is susceptible

TABLE IV

Leaf composition of three varieties of peach seedlings infected with *Meloidogyne incognita* or *Meloidogyne javanica*, planted February 28 and sampled September 15, 1949

Variety of peach and species of nematode	Inoculum (egg masses)	Weight of top and roots	Parts per million as determined spectragographically [1]										T.I.I.N
			Metal group Y				Non-metal group		Metal group Z				
	Number	G	Cu	Fe	Al	Mn	B	P	Na	Mg	Ca	K	
Lovell:													
None	0	111	24	620	1,160	5,680	62	3,740	105	6,660	15,660	27,300	61,01
M. incognita	123	109	26	535	1,200	5,860	64	3,700	135	5,170	16,000	36,670	69,36
,,	442	76	36	640	1,340	6,170	70	3,720	120	4,520	9,980	36,900	63,49
M. javanica	160	162	44	550	1,040	5,390	66	4,190	115	5,720	14,570	62,250	73,93
,,	515	97	28	630	1,230	6,100	68	4,090	110	5,370	11,100	42,000	70,72
Yunnan:													
None	0	140	25	560	1,160	4,550	58	3,230	100	4,900	10,480	20,530	45,64
M. incognita	123	187	26	575	1,080	4,510	62	3,610	115	4,540	11,350	26,830	52,70
,,	442	173	30	535	1,160	4,820	66	3,600	115	4,140	10,660	26,880	52,01
M. javanica	160	154	22	740	1,530	4,660	58	4,940	120	4,900	14,760	25,250	56,98
,,	515	104	36	560	1,190	5,850	58	3,600	100	3,400	8,330	28,750	51,87
S 37:													
None	0	124	44	680	1,300	5,170	84	4,000	120	5,200	13,000	20,500	50,10
M. incognita	123	149	26	425	900	4,800	56	3,420	105	5,590	16,200	27,100	58,62
,,	246	143	26	470	980	5,620	68	4,460	96	5,600	14,830	31,670	63,82
,,	442	134	34	545	1,100	4,700	84	3,700	100	5,470	13,390	27,070	56,19
M. javanica	160	155	26	590	1,200	5,280	68	4,210	115	5,620	15,270	28,410	60,79
,,	320	194	42	480	955	5,000	60	4,170	105	5,030	15,700	36,170	67,71
,,	515	122	30	580	1,130	5,160	74	3,700	132	4,940	12,030	38,000	65,78

[1]) Elements grouped according to chemical characteristics and physiological functions and arran within groups in general order of increasing concentration.

[2]) Total intensity of internal nutrition (sum of all elements determined).

to *M. javanica*, showed a decrease in the concentration of Mg with the maximum quantity of inoculum of this species. The records of other elements appear somewhat erratic.

Specht calculated the percentage deviation of elements in samples under consideration from a check or "normal" value and algebraically totaled these according to element groups (chart 1) as he did in analysis of alfalfa [24]). This grouping was used in the construction of table IV and chart 1. Chart 1 shows patterns of composition in leaves relative to the check samples and according to element groups. The shifts in the patterns of internal nutrient accumulations caused by different nematode treatments on different peach seedlings are indicated.

Both species of nematode reproduced on the variety Lovell. With heavy inoculum the general patterns of chemical composition are similar. *M. javanica* reproduced on the variety Yunnan and the heavy inoculum of this species gives a pattern of composition

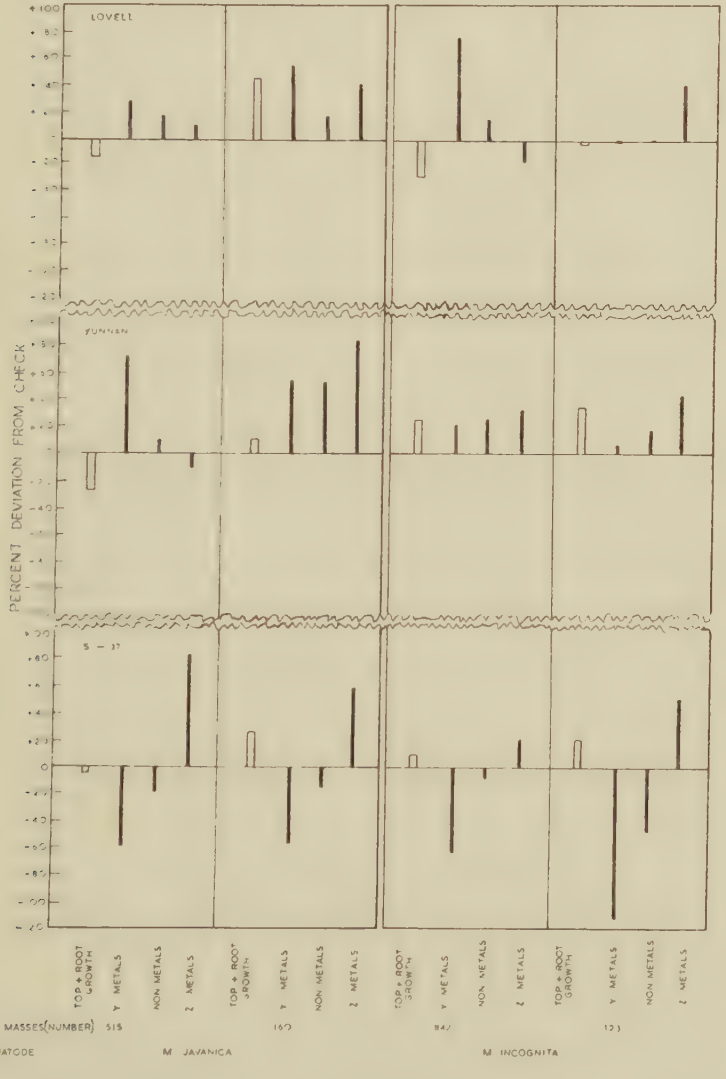

Chart 1. Growth and relative intensity of internal nutrient accumulation of groups of elements in leaves of peach seedlings infected with *Meloidogyne javanica* and *M. incognita*.

similar to the patterns of heavy inoculum of both species on Lovell. Neither nematode species reproduced to any extent on the variety S 37. All patterns of composition displayed by this variety are, therefore, different from those just discussed. They are similar to those of *M. incognita* on Yunnan. This similarity is reasonable since this species does not reproduce on Yunnan.

Since the growth of Lovell may be stimulated by a small quantity inoculation of *M. javanica* it is interesting that the element interrelations resulting from the two treatment levels are different (chart 1). The pattern of composition in the case of the low level inoculation resembles that produced by the same level on Yunnan on which stimulation likewise was indicated.

The data of table IV show that where growth stimulation occurred the accumulation of magnesium and calcium in the leaves was greater than where injury occurred. It is also noteworthy that potassium accumulation usually occurred regardless of whether the plant was stimulated or injured. Consideration of magnesium accumulation in Yunnan shows that *M. javanica* seems to exert a greater influence on the level of this element than *M. incognita*. The former species is also better adapted to this variety than the letter. Both species of nematodes reduced the magnesium level in Lovell; *M. incognita*, the species causing most injury to this variety, produced the greater change. Neither species of nematode reproduced well on S 37 and neither consistently reduced magnesium in this variety. Rather the nematodes tended to stimulate its accumulation. The same general behavior is noted for calcium in respect to the different nematode treatments on the different seedlings, the principal difference being that the calcium fluctuations are usually relatively greater than those of magnesium.

Copper, however, shows the reverse trend to magnesium and calcium in S 37, i.e., its accumulation is reduced regardless of nematode treatment. The same relation is observed for iron. Since these are enzyme elements this feature may be an extremely important factor in the resistant characteristic of S 37.

Deficiency of potassium, phosphorus, and nitrogen as a result of sugar beet infection with *Heterodera schachtii* Schmidt has long been known [22] [31] [32]. More recently Oostenbrink [21] reported nitrogen deficiency as a result of *Heterodera rostochiensis* Wollenweber parasitism of potato plants. Tarjan [26] reported nitrogen

and sodium deficiency in the roots of boxwood infected with meadow nematodes and increase of potassium in the roots of such diseased plants. Chlorosis as a result of nematode infections of plants is now a matter of common knowledge, but the explanation of the disturbing changes in plant nutrition as a result of nematodes can result in much controversy. Until such time as the complete nutrition of the plant and the nematode are both worked out, no single interrelationship can be fully understood. The stimulating effect of moderate quantities of nematodes is certainly a disturbing factor.

SUMMARY AND CONCLUSIONS

Various nematodes associated with decline and chlorosis of peach trees are discussed. Chlorosis appears to be due to deficiency of magnesium or iron or both. Either or both may be absorbed differentially by *Meloidogyne incognita* and *M. javanica*, root-knot nematodes.

Moderate quantities of *Meloidogyne incognita* and *M. javanica* sometimes cause significant increases in peach growth. Increase may be due to stimulation of root growth alone or to a combination of this factor with others not as yet completely understood.

The peach variety S 37 was relatively resistant to both these root-knot nematodes. Other root-knot nematode species exist and may or may not be capable of propagation on this variety.

Root-knot nematodes can cause significant damage to peach tree growth even when they are unable to propagate themselves on the roots of the variety. Breeding of resistant varieties, therefore, in no way lessens the importance of use of resistant cover crops in orchard plantings. Preplanting fumigation of the soil is likewise valuable even though the use of a resistant variety is planned.

In this investigation stimulation of growth of peach seedlings by nematodes was associated with greater accumulation of magnesium and calcium in the leaves than when injury occurred. In contrast to this tendency it is important that, in the most resistant variety, S 37, the accumulation of copper and iron was consistently reduced by inoculation with both nematodes.

Of all elements determined in the leaves only potassium usually showed an increase regardless of specific nematode treatment or seedling variety.

Consideration of the data from a nutrient-element balance standing-point revealed (1) that similar patterns of composition existed in the leaves of the seedlings having the greatest resistance to either species of nematode or to both species and (2) that the resistance was associated with a relatively lower accumulation of group Y than of group Z metals.

The information presented in this paper demonstrates why horticulturists plant pathologists, soil chemists, and nematologists may be at loggerheads over the cause and cure of plant diseases. Each one, seeing only a part of the picture, is apt to draw unfounded conclusions.

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REFERENCES

1) Anonymous. Report Chief of the Bureau of Plant Industry, U.S. Department of Agriculture **12** (1934).
2) Carrol, J., A study of the potato eelworm (*Heterodera schachtii*) in the Irish Free State. J. Helminthol. **11**, 137–156 (1933).
3) Carrol, J. and McMahon, E., Potato eelworm (*Heterodera schachtii*) investigations. Ibid. **13**, 77–90 (1935).
4) Chitwood, B. G., Root-knot nematodes. Part I. A revision of the genus *Meloidogyne* Goeldi, 1887. Proc. Helminthol. Soc. Wash. **16**, 90–104 (1949).
5) Chitwood, B. G., Ring nematodes (*Criconematidae*). A possible factor in decline and replanting problems of peach orchards. Proc. Helminthol. Soc. Wash. **16**, 6–7 (1949).
6) Chitwood, B. G. and Feldmesser, J., Golden nematode population studies. Proc. Helminthol. Soc. Wash. **15**, 43–55 (1948).
7) Chitwood, B. G., Host-parasite relationships of nematodes in root diseases. Phytopathology **39**, 502 (1949).
8) Chitwood, B. G., Root-knot nematodes. Part II. Quantitative relations of the root-knot nematode *Meloidogyne hapla*. Chitwood, 1949, with tomatoes, onions, and lima beans. Plant and Soil **3**, 47–50 (1950).
9) Christie, J. R., and Havis, A. L., Relative susceptibility of certain peach stocks to races of the root-knot nematodes. Plant Dis. Reptr. **32**, 510–514 (1948).
10) Cooper, H. P., Effects of energy properties of some plant nutrients on availability, on rate of absorption, and on intensity of certain oxidation-reduction reactions. Soil Sci. **69**, 7–39 (1950).
11) Curran, H. R., Brunstetter, B. C. and Myers, A. T., Spectrochemical analysis of vegetative cells and spores of bacteria. J. Bact. **45**, 485–494 (1943).
12) Day, L. H. and Tufts, W. P., Further notes on nematode-resistant rootstocks for deciduous fruit trees. Am. Soc. Hort. Sci. **37**, 327–329 (1939).
13) Day, L. H. and Tufts, W. P., Nematode-resistant rootstocks for deciduous fruit trees. Univ. Calif. Coll. Agr. Circ. **359**, 16 (1944).
14) Goodall, D. W. and Gregory, F. G., Chemical composition of plants as an index of their nutritional status. Imp. Bur. Hort. and Plantation Crops Com. **17** (July, 1947).
15) Havis, A. L., Chitwood, B. G., Prince, V. E., Cobb, G. S. and Taylor, A. L., Susceptibility of some peach rootstocks to root-knot nematodes. Plant Dis. Rptr. **34**, 74–77 (1950).
16) Hutchins, L. M., Nematode-resistant peach rootstocks of superior vigor. Proc. Am. Soc. Hort. Sci. **34**, 330–338 (1937).
17) Machmer, J. H., Soil fumigation for the control of the root-knot nematode in prach, fig, and grape plantings. Phytopathology **39**, 498 (1949).
18) McBeth, C. W. and Taylor, A. L., Immune and resistant cover crops valuable in root-knot-infetsed peach orchards. Proc. Am. Soc. Hort. Sci. **45**, 158–166 (1944).
19) Myers, A. T., Dyal, R. S. and Borland, J. W., The flame photometer in soil and plant analysis. Soil Sci. Soc. Am. Proc. **12**, 127–130 (1947).
20) Neal, J. C., The root-knot disease of the peach, orange, and other plants in Florida due to the work of *Anguillula*. U. S. Dept. Agr., Div. Ent. Bul. **20** (1889).
21) Oostenbrink, M., Het aardappelaaltje (*Heterodera rostochiensis* Wollenweber), een gevaarlijke parasiet voor de eenzijdige aardappel-cultuur. Veenman & Zonen, Wageningen, 230 pp. (1950).
22) Roemer, T., Handbuch des Zuckerrüben Baues, 366 Berl. (1927).

23) Shear, C. B., Crane, H. L. and Myers, A. T., Nutrient-element balance: A fundamental concept in plant nutrition. Proc. Am. Soc. Hort. Sci. **47**, 239–248 (1946).
24) Specht, Alston, W., The variation in mineral composition of alfalfa grown on Glenelg soil. Ph. D. thesis. Univ. of Md. (1948).
25) Steiner, G., Plant nematodes the grower should know. Soil Sci. Soc. Florida Proc. **4**–B (1942), 72–117 (1949).
26) Tarjan, A. C., A consideration of mineral nutrition of boxwood in relation to infection by meadow nematodes, *Pratylenchus* spp. J. Wash. Acad. Sci. **40**, 157–161 (1950).
27) Thatcher, R. W., A proposed classification of the chemical elements with respect to their functions in plant nutrition. Science, **79**, 463–466 (1934).
28) Tufts, W. P., Nematode resistance of certain peach seedlings. Proc. Am. Soc. Hort. Sci. **26**, 98–100 (1929).
29) Tufts, W. P. and Day, L. H., Nematode resistance of certain deciduous fruit tree seedlings. Proc. Am. Soc. Hort. Sci. **31**, 75–82 (1934).
30) Tyler, Joselyn, Plants reported resistant or tolerant to root-knot nematode infestation. U. S. Dept. Agr. Misc. Pub. **406** (1941).
31) Vanha, J., Die Enchytraeiden als neue finder der Zuckerruben, der Kartoffeln, und andere Landwirtschaftlichen Kulturpflanzen. Z. Zuckerind. Böhmen **17**, 157–182 (1893).
32) Wilfart, H. and Wimmer, G., Untersuchungen über die Wirkung der Nematoden als Ertrag und Zusammensetzung der Zuckerruben. Z. Ver. deut. Zucker-Ind. **53**, 1 (1903).

ERRATUM

Vol. III, p. 196: for "a mixture of 5 ml of HNO_3 (5 parts) and 4 ml of H_2SO_4 (6 parts)" read "5 ml of a mixture of HNO_3 (5 parts) and H_2SO_4 (6 parts)".